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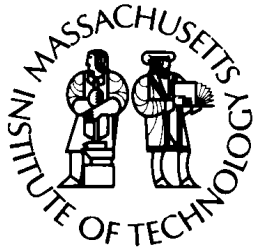

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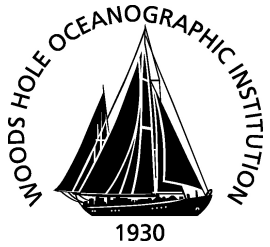

MASTER OF SCIENCE THESIS

Tropical Stony Corals Host Diverse Microbial Nitrogen Dynamics

by

Tamasi Tyler

September 2019

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by

Tyler Tamasi

A.B. Princeton University, 2015

Submitted in partial fulfillment of the requirements for the degree of

Master of Science

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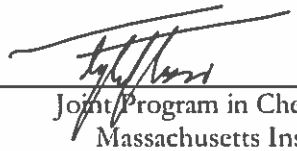

Joint Program in Chemical Oceanography
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July 10, 2019

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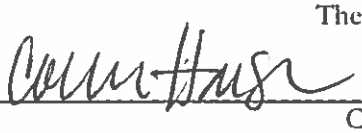

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Tropical Stony Corals Host Diverse Microbial Nitrogen Dynamics

by

Tyler Tamasi

Submitted to the Department of Earth, Atmospheric and Planetary Sciences and the Woods Hole Oceanographic Institution on July 10, 2019 in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemical Oceanography

ABSTRACT

Coral health hinges on an intricate relationship between the coral animal, photosynthetic algae of the family Symbiodiniaceae, and a microbial community of bacterial, archaeal, fungal, and viral associates collectively termed the coral holobiont. This holobiont maintains the nutrient balance of their symbiosis amid reefs' otherwise oligotrophic environment, including by cycling physiologically important nitrogen compounds. Nitrogen (N) fixation has been well quantified in corals in accounting for the source of their fixed N, but no complementary loss term had been directly detected. This pathway is important for understanding sources and sinks of nitrogen on reefs and how they may impact coral success.

Here we use ^{15}N-tracer experiments to produce the first measurements of nitrate reduction, nitrite oxidation, and nitrous oxide (N_2O) production in five species of reef-building corals in the Gardens of the Queen, Cuba. Nitrate reduction and nitrite oxidation are present in most species sampled, but ammonium oxidation is low potentially due to photoinhibition and competition with uptake. Coral-associated rates of N_2O production indicate potential for denitrification, although there are variations among species. The brain coral *Diploria labyrinthiformis* exhibits the strongest potential for denitrification based on elevated rates of nitrate reduction and N_2O production. This is in contrast with the elkhorn coral, *Acropora palmata*, which hosts minimal active nitrogen metabolism directly. Species sampled at multiple sites (*Porites porites* and *Orbicella faveolata*) showed similar trends among replicates and within genera (*Porites*).

We also examine the impact of light and dark treatments on coral-associated nitrogen cycling. We hypothesized that dark conditions would stimulate anoxia via decreased photosynthesis and, in turn, denitrification. Most species, including two of the genus *Porites*, display higher rates of nitrate reduction and nitrite oxidation in the dark. In contrast, the mountainous star coral *Orbicella faveolata* displays the highest rates of nitrate reduction and nitrite oxidation measured, but only under ambient light, implying at least partial mediation by phototrophic associates. These measurements directly confirm the potential of coral symbionts to conduct denitrifying metabolisms, which had previously been inferred by molecular evidence.

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1. Background

1.1. Coral reef biogeochemistry

Corals are foundational ocean engineers, building vast and complex reefs that support over 25% of marine life but occupy only 0.2% of the Earth's oceanic extent (Spalding and Grenfell, 1997; Miththapala, 2008). They thrive in shallow waters within a broad band from 30ºS to 30ºN of warm, sunlit, and relatively nutrient deficient, or oligotrophic, ocean waters (Kleypas *et al.*, 1999) (**Figure 1.1**). Despite this small geographic footprint, reefs support the livelihoods of up to 500 million people globally through fishing and tourism industries (Bouchon *et al.*, 2008; Knowlton, 2011) and provide more than half of the protein consumed by individuals in coastal tropical regions (UNEP, 2004). Alarmingly, the success of coral reefs has floundered in recent decades in the face of increasing ocean temperatures, pathogens, and pressures from overfishing, as well as changes in the global ocean's overall chemical landscape. These, along with regional factors like anthropogenic effluents, have led to the degradation of 33-50% of the world's reefs, placing a quarter of all marine species at risk of extinction (Hoegh-Guldberg *et al.*, 2017).

Coral health has classically been thought to hinge on the balance of a complex relationship between the coral animal and its mutually beneficial symbiosis with photosynthetic algae of the family Symbiodiniaceae. However, the coral-algal partnership depends on a complex microbial community of bacterial, archaeal, fungal, and viral associates, collectively termed the coral holobiont (Rohwer *et al.*, 2002). The holobiont maintains a delicate nutrient balance in order to support life in reefs' otherwise oligotrophic surroundings (Falkowski *et al.*, 2008; Bourne *et al.*, 2016). One such nutrient, nitrogen, is essential for the production of amino and nucleic acids and is a principal limit of ocean productivity. Naturally, then, nitrogen likely plays an important role in holobiont community function.

1.2. Coral health and nitrogen availability in tropical reef ecosystems

Tropical reef-building corals flourish despite the apparent constraints of their nutrient-poor environments (Darwin, 1889; Sammarco *et al.*, 1999). They are adapted to life at the precipice of resource availability and are aided by microbial associates that can continually supply new nitrogen to the ecosystem (Wegley *et al.*, 2007; Fiore *et al.*, 2010; Lema *et al.*, 2012, 2014). The marine nitrogen cycle is a finely tuned set of chemical transformations carried out by a diverse community including diazotrophic, nitrifying, and denitrifying microorganisms. Even though these microbes are ubiquitous among reef-building corals (Yang *et al.*, 2013), direct evidence of their function within the overall ecosystem has not been fully explored. The nitrogen-fixing microbes responsible for the conversion of otherwise inert N_2 to readily available ammonium (NH_4^+), may allow corals to thrive in otherwise oligotrophic environments by contributing nutrients to the coral ecosystem (Wiebe *et al.*, 1975); however, the roles of other nitrogen cycling pathways remain almost entirely unknown (Rädecker *et al.*, 2015) (**Figure 1.2**). Given the importance of nitrogen to cell growth and its scarcity on reefs, the coral nitrogen cycle is likely to span the suite of processes observed in world oceans at large and which have already been shown to be associated with other marine invertebrates (van Oevelen *et al.*, 2013).

1.3. Global mechanisms of the nitrogen cycle

Nitrogen is a globally important nutrient and proximal limit on marine photosynthesis across much of the oceans (William and Dodson, 1972; Eppley and Renger, 1974; Price *et al.*, 1991). The overall rates of fixed nitrogen sources and sinks into the oceans are predominantly set by two main microbial pathways: nitrogen fixation (input) and fixed nitrogen loss via denitrification (output) (Eugster and Gruber, 2012). Nitrogen fixation involves the cleavage of the strong dinitrogen triple bond to reduce N_2 and is attributed to autotrophic cyanobacteria in the surface oceans (Zehr, 2011) and non-cyanobacterial diazotrophs elsewhere (Zehr *et al.*, 1998, 2000; Farnelid *et al.*, 2011). Fixed nitrogen loss processes, on the other hand, occur in critically anoxic systems and are dependent on a

supply of organic matter (OM), increasing with rising OM supply (Ward *et al.*, 2008; Babbin *et al.*, 2014). Two main contributors to this nitrogen loss are canonical denitrification, the sequential reductions of nitrate (NO_3^-) to nitrite (NO_2^-) to nitric oxide (NO) to nitrous oxide (N_2O) and finally to dinitrogen gas (N_2), and anaerobic ammonium oxidation (anammox), the conversion of NH_4^+ and NO_2^- to N_2. Each of these pathways is carried out by microorganisms that possess specific genes encoding the enzymes to facilitate their functioning. Conventional nitrogenase, the enzyme that catalyzes nitrogen fixation, is encoded by the *nif* genes and is present across diverse lineages of prokaryotes (Young, 1992). Denitrification is catalyzed by the enzymes nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, encoded by the genes *nar*, *nir*, *nor*, and *nos*, respectively (Zumft, 1997). The enzymes that catalyze these nitrogen transformations display varying sensitivities towards oxygen (Bonin *et al.*, 1989), and so can be dependent on local O_2 microenvironments.

Broadly, the nitrogen cycle includes both aerobic and anaerobic pathways, with denitrification and anammox requiring low O_2 (Smethie, 1987; Strous *et al.*, 1997), N_2 fixation poisoned by the presence of O_2 (Postgate, 1982), but nitrification requiring it (Wezernak and Gannon, 1967). As a result, these processes are separated in space and time due to the inhibition of enzymes like nitrate reductase, the catalyst for the first step in denitrification, by oxygen (Korner and Zumft, 1989; Sabaty *et al.*, 1993). In the global oceans, a significant proportion of these anaerobic processes occur in oxygen minimum zones (OMZs), where oxygen concentrations are low enough for nitrate reduction to occur. These areas, like reefs, represent only a minute fraction (0.1%) of the ocean's volume (Codispoti, 2007), yet are responsible for 30-50% of total marine reactive nitrogen loss (Gruber and Sarmiento, 1997; Codispoti *et al.*, 2001). In these regions, denitrification is able to begin with nitrate reduction leading to characteristically high concentrations of nitrite and completed with the anaerobic reduction of this nitrite to gaseous N_2O and N_2 (Codispoti and Christensen, 1985; Thamdrup *et al.*, 2006). The diurnal

variations in oxygen concentrations observed within the mucus films and tissues of corals may permit similar suboxic metabolisms.

1.4. Oxygen variability on tropical coral reefs

Coral reefs exhibit dramatic fluctuations in dissolved oxygen concentrations following the diurnal cycle. Initial measurements on tropical reefs have shown dissolved O_2 concentrations ranging from just 40% saturation at dawn to 160% saturation during peak photosynthesis in the afternoon in the open seawater around corals (Sournia, 1976). Variability of dissolved oxygen levels can moreover be influenced by localized water-flow dynamics at the interface between corals and algae, increasing with faster advection (Brown and Carpenter, 2013). These fluctuations manifest at scales as large as entire reefs, which can display hourly O_2 fluxes between +2,000 mmol O_2 m^{-2} d^{-1} (net production) and –500 mmol O_2 m^{-2} d^{-1} (net consumption) (Long *et al.*, 2013), and as small as the gastric cavities of individual corals, which can vary between full saturation in the light to anoxia in the dark (Agostini *et al.*, 2012). Within coral boundary tissue layers these oscillations are still greater, with extremes that range from supersaturation in the day (373% air saturation) to anoxia at night, even as ambient water compositions remain constant (Shashar *et al.*, 1993). These variations persist to the submillimeter scale (Guadayol *et al.*, 2014). Even though these low oxygen conditions prime reefs for denitrification, the ability of tropical reef microbial communities to conduct nitrogen loss processes has received less attention than their neighboring reef carbonate sediments (Capone *et al.*, 1992), oyster reefs (Humphries *et al.*, 2016), and sponges (Hoffmann *et al.*, 2009).

1.5. Evidence of nitrogen cycling in corals and other invertebrates

Nitrogen-fixing bacteria in the coral holobiont have received much attention because of the importance of coral-associated diazotrophs in promoting Symbiodiniaceae populations via bio-available nitrogen in an otherwise oligotrophic environment (Lesser *et al.*, 2007; Lema *et al.*, 2012). Recently, mechanisms for nitrogen-fixing microbes to induce coral holobiont perturbation, and

subsequent coral stress and bleaching, have been identified (Pogoreutz *et al.*, 2017). Elevated temperatures and excess DOC, potentially from anthropogenic sources, may stimulate nitrogenase activity and promote the proliferation of coral-associated diazotrophs (Compaoré and Stal, 2010; Santos *et al.*, 2014; Cardini *et al.*, 2016). Counterintuitively, increased diazotrophy relieves Symbiodiniaceae from its typically nitrogen-limited cell division and upsets the nutrient balance of coral-algae symbiosis, leading to symbiont expulsion (Falkowski *et al.*, 1993; Dubinsky and Jokiel, 1994). Although even these elevated nitrogen fixation rates in corals are about a magnitude lower than those found in reef sediments and on bare rock (Cardini *et al.*, 2016), nitrogen fixation within the coral holobiont likely plays a key role in overall coral health because of proximity to the animal itself.

While there have been multiple studies of nitrogen fixation pathways in tropical reef-building corals, there have been no direct rate measurements of fixed N loss despite the identification of microbial communities associated with nitrate reduction (Wegley *et al.*, 2007). In addition, diverse bacterial nitrite reductase genes have been found in tissue from the orange cup coral *Tubastrea coccinea*, and have also been detected in bacterial associates of the soft coral *Alcyonium gracillimum*; these results imply the presence of diverse nitrite-reducing symbionts in corals (Yang *et al.*, 2013). While suggestive, the genetic evidence highlights a need for direct rate measurements of denitrification in tropical reef-building corals in order to confirm the activity of suboxic metabolic pathways.

Whereas a detailed set of direct rate measurements has not been conducted in tropical reef-building corals, evidence from cold-water corals suggests a role for nitrogen metabolisms beyond fixation. In contrast to tropical corals, cold-water corals thrive in deeper, darker, and more nutrient-rich waters, and are usually devoid of algal symbionts (van den Beld *et al.*, 2017). Cold water corals have been shown to exhibit a fuller nitrogen cycle comprising fixation, nitrification, and denitrification (Kiriakoulakis *et al.*, 2006; Middelburg *et al.*, 2015; van Oevelen *et al.*, 2018). Incubations with the cold-water species *Lophelia pertusa* directly detected nitrogen fixation, nitrification, ammonium assimilation,

and canonical denitrification (Middelburg *et al.*, 2015). Nonetheless, the substantial differences in light, temperature, O_2, NO_3^-, and other environmental variables preclude extrapolation from cold-water corals to tropical corals without direct measurements of nitrogen cycling rates.

In tropical reefs, sponges have been shown to harbor a markedly complex and tightly interwoven microbially-driven nitrogen cycle (Hoffmann *et al.*, 2009; Fiore *et al.*, 2013). Like corals, sponges host communities of physiologically diverse microorganisms (Hentschel *et al.*, 2006); however, they are also capable of modulating their internal oxygen concentrations by regulating their pumping behavior in order to support both aerobic and anaerobic microbes (Hoffmann *et al.*, 2005) and diverse nitrogen metabolisms (Schläppy *et al.*, 2010). In oligotrophic tropical reef environments, which neither conserve nor export significant amounts of inorganic nutrients (Wafar *et al.*, 1990), active nitrogen transformations are vital for maintaining a highly productive biological environment in the absence of ambient nutrient availability. The presence of these pathways in other organisms, as well as oxygen fluctuations that would prime microzones for denitrification, make exploring the capacity of tropical corals to exhibit similar complexity promising.

1.6. Goals of this study

The dynamics of the coral-associated nitrogen cycle remain unknown due to a lack of direct rate measurements on tropical reef-building species. With a sudden pulse of nutrients, like that of anthropogenic effluent or a pulse of ammonia excreted from fish (Randall and Wright, 1987), the relative importance of these pathways would be expected to temporarily shift in order to return to an overall balance (Burkepile *et al.*, 2013; Rädecker *et al.*, 2015). Coral health hinges on the complex and dynamic relationship among members of the holobiont; microbial nitrogen cycling may play a crucial role in maintenance of the seawater and boundary layer chemistry underlying the stability of the coral holobiont. The network of microbial nitrogen metabolisms is an especially important consideration in modern reefs, as anthropogenic nutrient influxes, warmer temperatures, and decreased pH can alter

the nitrogen speciation of the bulk water. However, without a base understanding of the tightly coupled steps involved in these pathways, further projections regarding the relationship between nitrogen and coral health will remain abstract. The first step toward this goal is the quantification of a baseline coral nitrogen cycle for multiple individual coral species. This thesis provides the first measurements of nitrate reduction to nitrite, nitrite oxidation to nitrate, and nitrite reduction to nitrous oxide and dinitrogen in the globally important tropical reef-building corals.

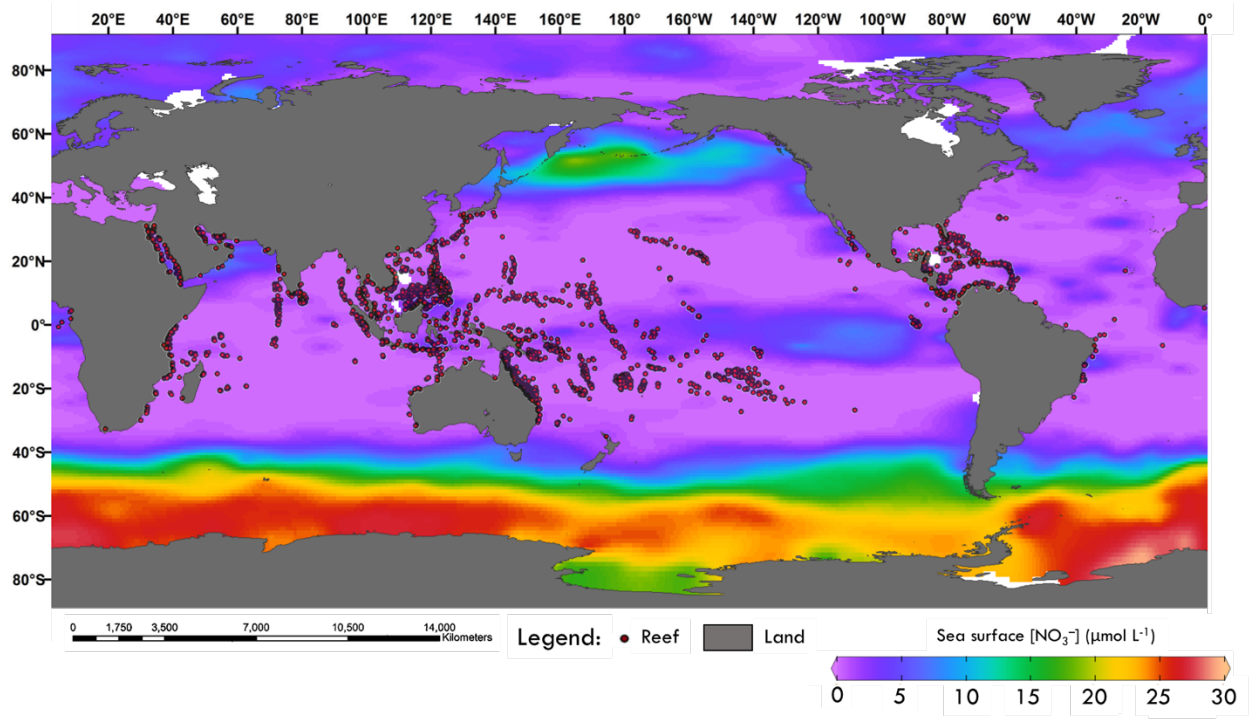

Figure 1.1: Global distribution of tropical coral reefs (circles) overlaid on sea surface nitrate concentrations. Corals thrive in sunlit, oligotrophic seas that are noticeably lacking in the nutrients – particularly nitrogen compounds – that are characteristic of more productive regions. Figure adapted from NOAA Global Coral Database (UNEP-WCMC, 2010) and GLODAP version 2 (Key *et al.*, 2015).

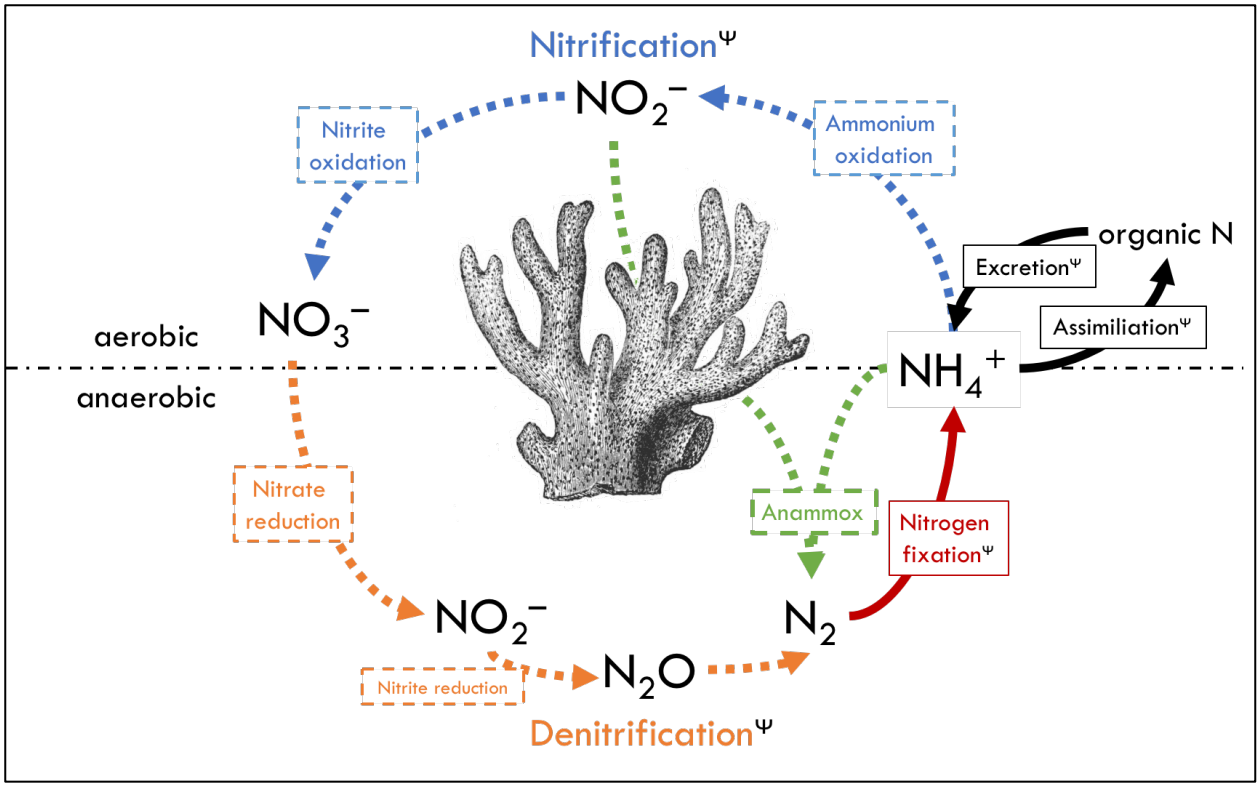

Figure 1.2: Schematic of a hypothetical, simplified nitrogen cycle for tropical reef-building corals prior to this work. Dashed arrows demark rates that have not yet been quantified, whereas solid arrows represent those that have been characterized for tropical Scleractinian corals. $^{\Psi}$These rates have been measured for cold-water corals.

2. Methods

2.1. Study area

The Gardens of the Queen (Jardines de la Reina) is a pristine Caribbean reef environment located along the southern coast of Cuba, sheltered historically due to its remote nature from the Cuban coastline and protected officially since 1996 by the Cuban government. It hosts lower degrees of local pollution, fishing pressures, and other anthropogenic influences than are present in other areas of the Caribbean (Gardner *et al.*, 2003; Knowlton, 2011; Thampi *et al.*, 2018). Many reef ecosystems elsewhere in the region, such as Jamaica and the Florida Keys, have been heavily impacted by these human influences and subsequent coral disease outbreaks (Eakin *et al.*, 2010; Jackson *et al.*, 2012). The Gardens of the Queen stands in stark contrast, as an extensive national system of marine protected areas that has resulted in a notably well-preserved ecosystem.

Additionally, the Gardens of the Queen afforded our study the opportunity to investigate species, like *Acropora palmata*, that have experienced precipitous population declines elsewhere in the Caribbean (Joyner *et al.*, 2015). Fish and coral densities are highest in the central regions of the Gardens and slightly lower at the NW and SE-most parts, where highly-regulated fishing practices are permitted on a limited basis (Valdivia *et al.*, 2017). Seven sites were chosen for sampling from a variety of reef types, and one coral species was chosen per site based on prevalence and availability (**Figure 2.1**).

2.2. Sample site characteristics

Nitrate, nitrite, and ammonium concentrations at each of the sample sites were measured from water samples (reef depth, 30 mL) using colorimetric methods (Strickland and Parsons, 1972; Hydes *et al.*, 2010) (**Figure 2.2**). Total nitrogen, which includes the total organic and inorganic nitrogen, was determined using UV photo-oxidation (Walsh, 1989; Hansell and Carlson, 2000). At each site, scuba divers also conducted benthic surveys to estimate the percent coral cover relative to

other reef substrates such as macroalgae, sponges, rocks, and sand. To do so, the composition (in cm) of each substrate using a transect tape was recorded at 1 m intervals over a total distance of 10 m, and this was replicated 8-10 times over the reef. Chlorophyll concentrations were determined by extraction of samples in 90% acetone at 20ºC for 24 h (Holm-Hansen *et al.*, 1965) and quantified using an AquaFlor fluorometer.

2.3. Coral sampling protocol

Corals were collected at each site from a depth of 9-14 meters from a single colonial head per site. Coral fragments for incubation were collected from each colony by hammer and chisel. These fragments were placed in sterile WhirlPaks and returned to the surface where they were kept at seawater temperature until introduction to the incubation vessels. Coral pieces were largely clean and lacking endolithic algae or sponges, which was confirmed by visual inspection. Pieces were 1 to 10 cm^3 in volume, depending on the species sampled, and generally did not vary in size within an incubation by more than a factor of 4.

2.4. Coral incubation vessels and experimental design

The corals were transferred into sterile on-deck incubators within 2 hours of removal from the reef. Each vessel was a 12 cm x 10 cm x 3 cm (360 mL) rigid, chemically inert, polycarbonate container selected for its relative clarity across the UV and visible light spectrum (Pelican Products, USA) (**Figure 2.3**). The containers were modified to include a Viton rubber gasketed bulkhead compression port fitted with a grey chlorobutyl rubber septum. The port allowed each container to be filled completely without residual air bubbles and also provided a means of injecting ^{15}N tracers. Each container was previously submerged to ensure a complete seal using colored dyes; none allowed water exchange during the course of the 24-hour quality control test.

To set up each incubation, at least one coral piece was placed in a container that was then filled completely with reef water. This water had been collected directly from the reef where the

parent coral colony had been situated using a 10 L Niskin bottle. Using ambient reef water ensured that the chamber contained a similar chemical, microbiological, and metabolomic profile to the corals' natural environment and so would be most indicative of on-reef conditions. These chambers, filled with reef seawater and corals, were incubated for approximately 24 hours at reef temperatures maintained by continuous flowing surface seawater. In all, the time from the collection of corals on the reef to the start of the incubation via inoculation was approximately 2 to 3 hours.

2.5. ^{15}N incubation scheme

Once coral pieces were in their incubation vessels, each container was amended to a final concentration of ~1 μmol L^{-1} of one of three tracer species: $^{15}NO_3^-$, $^{15}NO_2^-$, or $^{15}NH_4^+$. The ^{15}N tracers were made from $K^{15}NO_3$, $Na^{15}NO_2$, or $^{15}NH_4Cl$ salts, respectively (>99%, Cambridge Isotope Laboratories). In addition, natural abundance additions of the complementary DIN compounds were introduced to serve as carriers and to standardize inoculation amendments across experiments. As such, 1 mmol L^{-1} stock solutions of the appropriate $^{14/15}$N compounds were mixed to make three combined tracer solutions: each contained one of three ^{15}N labels and the two ^{14}N carriers. Final concentrations were attained by a single injection of 1 mL of the combined tracer solutions into the incubation vessels via a 22G needle through the septated port. The $^{15}NO_3^-$ tracer assessed rates of reduction to NO_2^-, N_2O, and N_2 (denitrification), the $^{15}NO_2^-$ tracer the rate of its oxidation to NO_3^- and reduction to N_2O and N_2, and the $^{15}NH_4^+$ tracer the rates of oxidation to NO_2^- (nitrification), N_2O (nitrifier denitrification) and N_2 (anammox) (**Table 2.1**).

Table 2.1: Experimental setup and measurement scheme

^{15}N tracer (1 μM)	^{14}N carriers (1 μM)	Measurement	Processes
NO_3^-	NO_2^-, NH_4^+	NO_2^-, N_2O, N_2	Nitrate reduction to nitrite; N_2O and N_2 production (denitrification)
NO_2^-	NO_3^-, NH_4^+	NO_3^-, N_2O, N_2	Nitrite oxidation to nitrate (nitrification); N_2O and N_2 production (denitrification)
NH_4^+	NO_3^-, NO_2^-	NO_2^-, N_2O, N_2	Ammonium oxidation to nitrite (nitrification); N_2O (nitrifier denitrification) and N_2 production (anammox)

In each set of experiments, six incubations were performed per ^{15}N label for a total of 18 individual incubations per site. Twelve of these contained corals from the designated site (four per ^{15}N tracer), and an additional six incubations were seawater only controls to measure ambient DIN cycling rates in reef water alone for comparison.

At each site, samples were also collected to serve as initial timepoints; one was collected for each ^{15}N tracer experiment. Each was amended to a final concentration of 1 μmol L^{-1} $^{15}NO_3^-$, $^{15}NO_2^-$ or $^{15}NH_4^+$ and 1 μmol L^{-1} of the complementary ^{14}N species with the same isotopic tracer mixture injected into the experiments. This inoculated reef water was then immediately split between each of two sample bottles: a 30 mL acid-washed high-density polyethylene (HDPE) bottle and a 30 mL glass serum bottle, both of which had been pre-treated with a 50 μL aliquot of 7 M $ZnCl_2$ as a preservative. 25 mL was injected into each bottle and the glass serum bottle was then crimped closed. This gas-tight sample was used for the isotopic measurement of dissolved N_2O and N_2, while the sample preserved in the HDPE bottle was used for the measurement of dissolved NO_3^- and NO_2^-.

Incubations were separated into one of two light treatments in order to investigate the impact of irradiance on DIN cycling rates. Half of the incubations were covered with dark mesh to reduce incident light levels at the surface and better approximate ambient illumination at the coral's natural depth – this is referred to as the "ambient light" treatment – while the other was covered with opaque, black plastic in order to estimate the effect of photosynthesis on the coral's biogeochemical nitrogen cycling profile – the "dark" treatment. Corals in the "ambient light" treatment were otherwise subject to the normal diurnal cycle. Light levels were confirmed using a Vernier photosynthetically active radiation (PAR) sensor and indicated that the mesh used in our "ambient light" treatment attenuated incident light by 80%, thus approximating the light levels that corals would receive at a depth of 8-10 m (Barron *et al.*, 2009).

Corals were incubated for a 24-hour period so as to capture a full diurnal cycle as well as to allow enough time for processing of the $^{15/14}N$ inocula, but also provide a short enough window to minimize bottle effects typically associated with lengthy incubations. At the end of each incubation, containers were opened and water samples were collected from each vessel. A total of 50 mL was split between HDPE and glass serum bottles as in the collection of the initial timepoint samples. Once water samples had been taken, corals were removed from the vessels, wrapped in aluminum foil, and frozen at -80°C until processing.

2.6. Coral surface area determinations and normalization metrics

Estimated dry tissue weights, volumes, polyp counts, and three-dimensional surface areas were determined for each coral sample as normalization metrics for the incubations. First, each coral piece was airbrushed (Paasche Model H) at 80 to 100 psi with a 0.2 μm filter sterilized phosphate-buffered saline (PBS) solution to remove coral mucus and tissue. The slurry was vortexed for 5 min to homogenize and a fraction of it was then dried and weighed; the rest was centrifuged at 20,000 xg for 20 min into a pellet and preserved at -80ºC for later molecular use outside the scope of this thesis. An estimate of the original tissue mass was then back-calculated based on the weight of the dried portion in comparison to the weight of the total tissue and PBS slurry volume. Cleaned coral skeletons were measured for surface area, volume, and polyp counts, which were generally more reliable than the dry weight estimates as the complete homogenization of the slurry proved difficult.

Three-dimensional coral surface areas were estimated using the aluminum foil method (Marsh, 1970), in which each cleaned coral skeleton is wrapped in a monolayer of aluminum foil and the resultant mold is weighed. Foil was used to cover only the area of coral that would have had live tissue, and all measurements used a single roll of standardized foil. Although widely adopted, this protocol does not capture finer details of structure inside of polyps and of species with particularly complex morphologies, like *Orbicella faveolata*, which have many small tube-like polyps. Nevertheless,

this is a commonly used means for capturing relative surface area among individuals (Naumann *et al.*, 2009; Veal *et al.*, 2010). Polyp density was determined by individually counting each polyp on each coral piece via visual inspection and then normalizing by the surface area estimates. The uniformity of polyp densities within a species lends credit to the aluminum foil determination of surface area as the most reliable indicator of sample size. Coral volumes were measured as water displacement in 25 mL or 50 mL graduated cylinders, depending on coral fragment size.

2.7. ^{15}N Rate Measurements using an Isotope Ratio Mass Spectrometer (IRMS)

2.7.1. Isotopic composition determinations

The isotopic compositions of dissolved nitrogen species, $^{15}N/^{14}N$, were determined using a continuous flow IsoPrime 100 isotope ratio mass spectrometer (IRMS). Ammonium oxidation to nitrite and nitrate reduction to nitrite samples were measured after conversion of sample nitrite to N_2O in a buffered sodium azide solution (McIlvin and Altabet, 2005; Tu *et al.*, 2016). A 1:1 mixture of 2 M sodium azide and 20% glacial acetic acid was added to each sample in a gas-tight and crimp-sealed 20 mL glass vial (Restek). Each vial received 0.1 mL azide working reagent per 1mL of sample and was allowed to react for 1 hour at room temperature. Samples were then neutralized with 0.1 mL 6 M NaOH per 1 mL of sample before introduction into the IRMS.

For experiments targeting nitrite oxidation to nitrate, excess labeled nitrite was first removed by sulfamic acid addition (Lipschultz *et al.*, 1990; Granger *et al.*, 2006). Sample aliquots were treated with 5% sulfamic acid (100 μL per 1 mL sample volume) for 1 h before neutralization with 4 M NaOH (80 μL per 1 mL sample volume). The treated samples were then injected into vials containing washed 10x cell suspensions of partial denitrifier culture *Pseudomonas aureofaciens* (Sigman *et al.*, 2001), which quantitatively reduced the nitrate in solution to N_2O. Due to the presence of $ZnCl_2$ in our samples, a greater ratio of *Pseudomonas aureofaciens* to sample (3.2 mL per 10 mL sample) was used than is typical in marine studies to overcome possible toxicity effects.

N_2O and N_2 production rates from each incubation were measured directly in the samples stored in gas-tight, glass serum bottles. N_2 measurements were performed first by sampling 1 mL of the headspace into a Hamilton gastight syringe and injecting directly into the IRMS. Due to the substantial headspace in the serum bottles, the detection limit of N_2 production was 1 μmol L^{-1} day^{-1} (40-200 nmol cm^{-2} day^{-1}). The same serum bottle was then sampled for ^{15}N-N_2O production by sparging with He at a rate of 100 mL min^{-1} for 15 minutes. The liberated N_2O was concentrated in a liquid nitrogen cryotrap before injection on the IRMS. Rates were calculated based on the difference between the initial and final timepoint measurements.

2.7.2. Carrier considerations

Due to the low DIN concentrations in our samples, preliminary isotopic measurements on the IRMS lacked sufficient quantities of product N_2O for reliable peak signals. In order to improve the fidelity of the peak signals measured, and the resulting isotopic compositions, an additional NO_3^- or NO_2^- carrier was added during processing in order to increase the final amount of N_2O in each sample to between 10-20 nmol. The isotopic $\delta^{15}N$ composition of this carrier was well characterized (-1.7‰ for the internal sodium nitrite standard and -1.8‰ for the USGS 34 potassium nitrate standard), and all carrier amounts were weighed gravimetrically to minimize errors. In the case of dissolved N_2O analyses, the carrier derived N_2O was added in-line to the He stream by daisy-chaining the sealed sample volumes together with the carrier N_2O produced by the sodium azide method from the internal nitrite standard.

2.7.3. Normalization of rates

Coral surface areas were used to normalize initial rate measurements from each incubation to a rate per surface area of coral tissue:

$$R_{normalized} = V * \frac{R_{coral} - R_{SW}}{SA} \qquad \textbf{Equation 2.1}$$

where $R_{normalized}$ is the surface area normalized transformation rate (nmol cm^{-2} day^{-1}) attributable to coral-associated nitrogen metabolism. Normalization by surface area is important because of the role of coral tissue, and the mucus it secretes, in harboring the coral microbial community. This determination was used to scale the detected rates by the active holobiont area to enable comparison across species and sites. As all corals were incubated while submerged in reef seawater, rates from coral incubations (R_{coral}) were assumed to be a combination of both coral and seawater transformation rates (R_{SW}). Thus, the rates from SW-only incubations were subtracted during calculation of coral-associated rates in order to compare the nitrogen cycling potential among coral species absent contributions from the surrounding reef water.

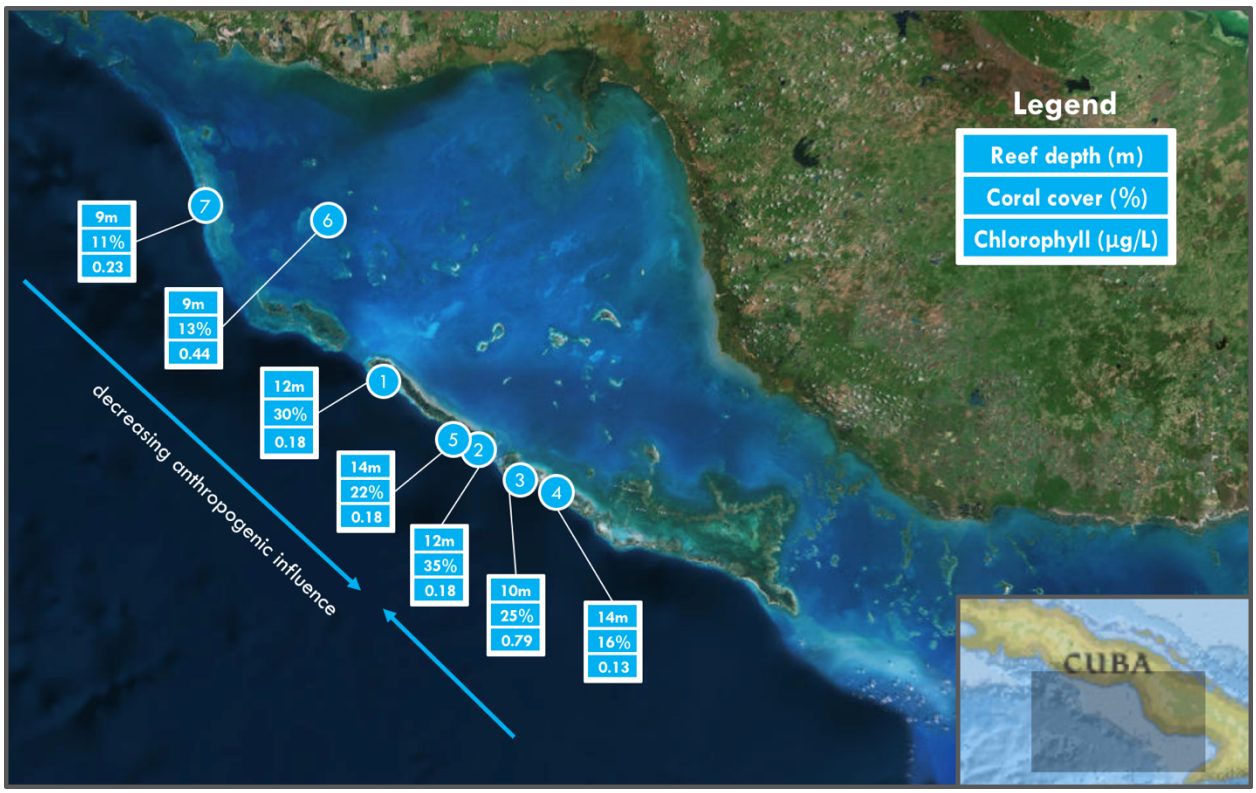

Figure 2.1: Gardens of the Queen, Cuba, sampling sites and associated reef characteristics. Coral cover was highest in the central sampling sites. Satellite map (DigitalGlobe) acquired via ArcGIS (ESRI, 2011).

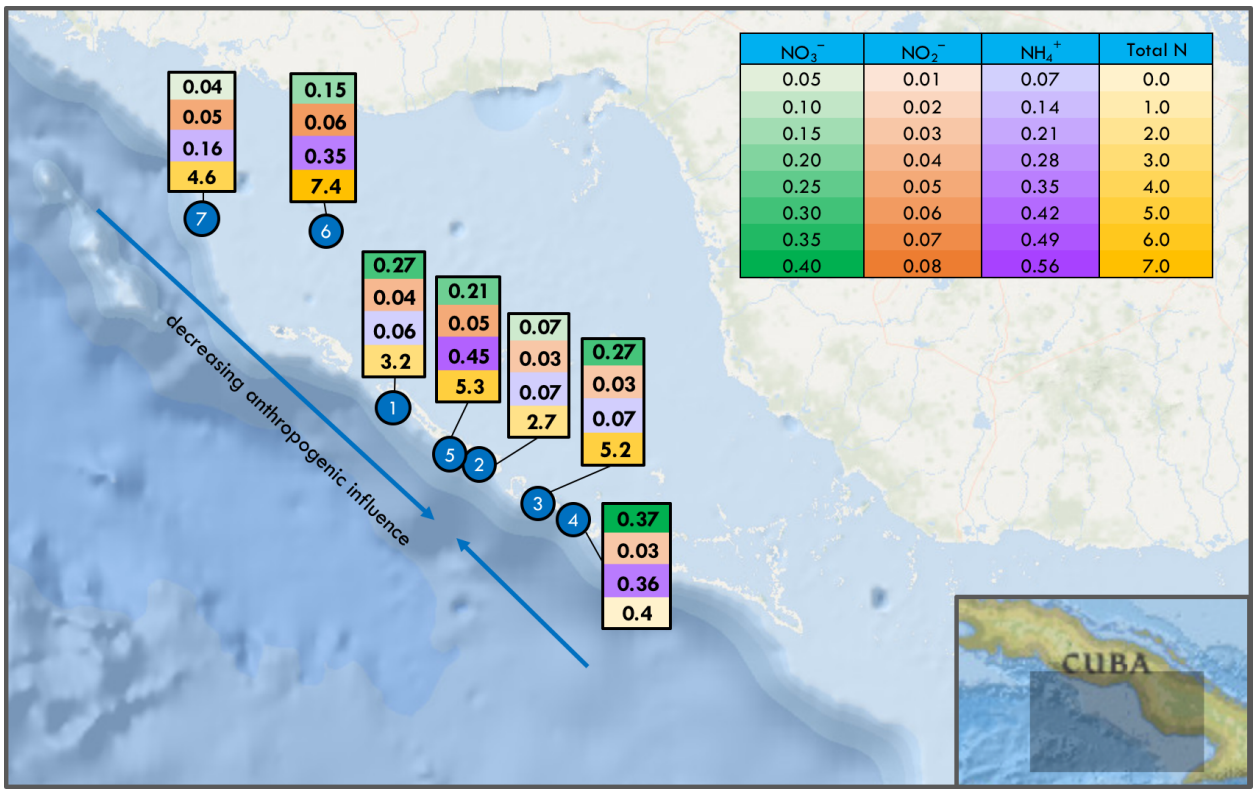

Figure 2.2: Inorganic and total (organic + inorganic) nitrogen concentrations (µmol L^{-1}) measured at reef depth at the sites where corals were sampled for the incubation experiments. *Porites astreoides* was sampled at site 1, *Porites porites* was sampled at sites 2 and 3, *Orbicella faveolata* was sampled at sites 4 and 7, *Diploria labyrinthiformis* was sampled at site 5, and *Acropora palmata* was sampled at site 6. Topographic map (DeLorme) acquired via ArcGIS (ESRI, 2011).

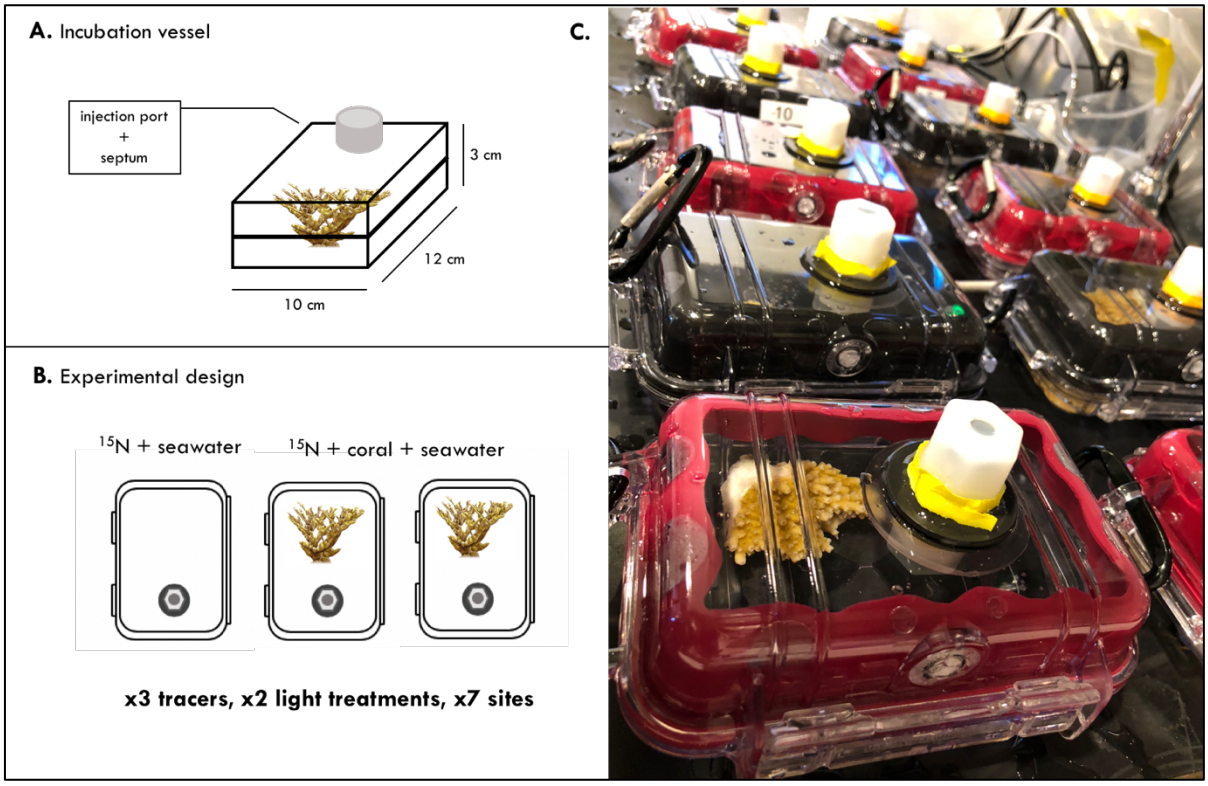

Figure 2.3: Incubation containers and experimental set up. **A:** Individual polycarbonate incubation vessels were 360 mL in volume and were opened individually via a hinge on one side. A rubber septum in the top allowed for injection of tracer. **B:** Experimental design, with 18 incubation vessels used at once. Six corals were incubated per tracer (3 per each of either light or dark treatments), with 2 out of 3 vessels containing coral pieces and the third incubating unfiltered reef water. **C:** Picture of incubation vessels filled with coral pieces, *A. palmata* in this photo.

3. Results

3.1. Coral species and sites sampled

A variety of coral species were studied in the Gardens of the Queen across multiple genera and morphologies (**Figure 3.1**). At each site, one species was sampled according to the availability and abundance of coral pieces to minimize impact of destructive sampling. Overall, coral cover was higher at these sites (22% on average) as compared with the rest of the Caribbean (currently estimated at 10-16%) (Gardner *et al.*, 2003; Schutte *et al.*, 2010). Sites 1, 2, 3, and 5, which were in the central archipelago, had greater coral cover (22-35%) than those at the fringes (11-16%).

Two species from the genus *Porites* were sampled. These corals typically have a hemispherical shape and slow growth rates. They tend to be long lived, up to hundreds of years (Ritchie and Smith, 1997). The Caribbean *Porites astreoides*, commonly known as mustard hill coral, may grow up to 1 m but tends to form more numerous, smaller colonies. They occur in shallow, tropical reef environments, reef slopes, and lagoons (Wallace et al., 2007). *Porites porites*, or finger coral, is formed from tiny polyps that branch outward to form irregular patches of short, finger-like lobes (van der Land, 2012).

The other species sampled were all from different genera. *Diploria* is a genus of massive reef-building corals represented by a single species, *Diploria labyrinthiformis*, which is commonly known as grooved brain coral. During the experiments, this coral produced qualitatively more mucus than any of the *Porites* and *Acropora* corals, and this was confirmed by visual inspection.

Orbicella faveolata, or mountainous star coral, was formerly classified as *Montastraea faveolata* and is a common reef-building coral in the Caribbean. It is now listed as endangered by the International Union for Conservation of Nature (Aronson *et al.*, 2008). Colonies are typically large, forming a mound with small bumps and undulations. It is often the most abundant coral species on fore reef slopes between 10 and 20 m (Vermeij *et al.*, 2006). As with *D. labyrinthiformis*, this species produced

qualitatively more mucus than any of the *Porites* and *Acropora* corals during the experiments, and a similar amount to *Diploria*, again confirmed by visual inspection.

Acropora palmata, or elkhorn coral, is structurally complex with many large branches that create habitats for a variety of reef species. *A. palmata* colonies are fast-growing, with an average growth rate of 5-10 cm per year (Gladfelter *et al.*, 1978). Recently, these corals have lost over 70% of their coral cover in the Caribbean due to white pox disease, a pathogen transmitted via anthropogenic outflows (Sutherland *et al.*, 2010), and are also listed as endangered.

3.2. Coral nitrogen transformation rates versus seawater

Rates of nitrate reduction to nitrite, nitrite oxidation to nitrate, and ammonium oxidation to nitrite were detected in these experiments, but not in all incubations (**Figure 3.2**). Measured coral-associated rates of nitrate reduction (~1-14 nmol cm^{-2} d^{-1}) and nitrite oxidation (~1-10 nmol cm^{-2} d^{-1}) were greatly in excess of ammonium oxidation (<1 nmol cm^{-2} d^{-1}) and all transformation rates in the dark treatments were generally higher than rates in the light treatments, except for incubations of *O. faveolata*. The relative magnitude of nitrate reduction versus nitrite oxidation varied by species, and nitrite oxidation was greater than nitrate reduction in *D. labyrinthiformis*.

Most nitrate reduction and nitrite oxidation rates were greater than their associated seawater only (SW) rates (**Figure 3.3**), implying metabolic nitrogen cycling activity concentrated by the coral and coral-associated microbial community. In general, average SW rates were near zero for nitrate reduction (0.1 ± 0.3 nmol L^{-1} d^{-1}) and ammonium oxidation (0.2 ± 0.5 nmol L^{-1} d^{-1}) and less than 10 nmol L^{-1} d^{-1} in all incubations. Average SW rates for nitrite oxidation incubations were 3.0 ± 1.7 nmol L^{-1} d^{-1}, which was higher than for the other SW rate measurements. For incubations where the coral-inclusive transformation rate was roughly equivalent to the ambient SW rate, as was the case for light nitrite oxidation by *P. astreoides* and dark N_2O production (from $^{15}NO_2^-$ injection) by *A. palmata,* the observed enrichments are assumed to be due to the reef water microbial community rather than coral-

associates since each chamber contained a mixture of coral and ambient reef water. The contribution of SW rates is thus subtracted during calculation of normalized rates in order to compare the nitrogen cycling potential among coral associates absent contributions from the surrounding reef water.

Surface area normalized rates (**Figures 3.4**) represent the transformation rate attributable to the coral community (coral-associated rate minus ambient seawater rate; see **Methods**) per exposed tissue surface. This normalization allows comparison between disparate coral morphologies and insight into the nitrogen cycling potential of the microbial community encapsulated in each species' tissue, which covers the outer surface of their calcium carbonate skeletons, and mucus, which is secreted across this interface.

3.2.1. *Porites astreoides*

Porites astreoides was sampled at site 1, and similar rates were detected for nitrate reduction (2.5 ± 0.8 nmol cm^{-2} d^{-1}) as for nitrite oxidation (1.7 ± 0.04 nmol cm^{-2} d^{-1}). For both transformations, the dark rate was higher than the light rate by ~2 nmol cm^{-2} d^{-1}, although light nitrite oxidation was not observed in excess of the ambient SW rate. Ammonium oxidation rates were low overall (0.1 ± 0.1 nmol cm^{-2} d^{-1}), and the ammonium oxidation rate under ambient light was roughly equivalent to the SW rate.

3.2.2. *Porites porites*

Porites porites was sampled at sites 2 and 3, with consistent patterns detected between sites. For example, at both sites nitrate reduction (2-6 nmol cm^{-2} d^{-1}) and nitrite oxidation (1-3 nmol cm^{-2} d^{-1}) were greater than ambient SW rates, although nitrite oxidation in ambient light was approximately equivalent to the SW rate at site 3. At both sites, dark transformation rates were greater than light transformation rates and ammonium oxidation was negligible (≤0.01 nmol cm^{-2} d^{-1}). In addition, nitrate reduction was greater than nitrite oxidation at both sites, and all rates were higher at site 3 than at site 2.

The normalized rates for *P. porites* show that dark nitrate reduction at site 2 (5.8 ± 4.0 nmol cm^{-2} d^{-1}) was in the range of nitrate reduction at site 3 (2.4 ± 1.2 nmol cm^{-2} d^{-1}). The light nitrate reduction at site 2 was also approximately equal to the light nitrate reduction rate at site 3 once normalized (all were ~2 nmol cm^{-2} d^{-1}). At site 2, nitrite oxidation in the dark was not statistically different from the other nitrite oxidation rates after normalization considering its large variability.

3.2.3. *Diploria labyrinthiformis*

D. labyrinthiformis was sampled at site 5, and the observed nitrite oxidation and nitrate reduction rates were both greater than in the SW incubations. Ammonium oxidation was again negligible. Unlike in the other species, the nitrite oxidation rates measured for *D. labyrinthiformis* were greater than nitrate reduction rates (6.7 ± 1.0 nmol cm^{-2} d^{-1} versus 2.1 ± 0.2 nmol cm^{-2} d^{-1} respectively), although rates overall were still higher in the dark treatment than in the light treatment for both. *D. labyrinthiformis* displayed the highest rates of dark nitrite oxidation of any species (9.4 ± 1.8 nmol cm^{-2} d^{-1}), and the greatest light nitrite oxidation rate (3.9 ± 0.9 nmol cm^{-2} d^{-1}) aside from *O. faveolata*, although with less variability between replicates than for *O. faveolata*.

3.2.4. *Orbicella faveolata*

Orbicella faveolata were sampled at sites 4 and 7, with coral-associated nitrite oxidation and nitrate reduction rates measured in excess of the SW rates for the light treatments at both locations. Dark nitrate reduction was observed (0.6 ± 0.6 nmol cm^{-2} d^{-1}) in addition to the highest average nitrate reduction in the light (12.8 ± 12.0 nmol cm^{-2} d^{-1}). There was high variability between individual corals incubated for this species, particularly at site 7 (24.8 nmol cm^{-2} d^{-1} compared with 0.8 nmol cm^{-2} d^{-1} between light nitrate reduction replicate incubations), but the general trend of high nitrate reduction and nitrite oxidation in the light treatment was consistent between sites. Ammonium oxidation was not detected.

Normalization confirmed that rates of nitrate reduction and nitrite oxidation in the light were the highest of any species sampled per tissue surface. These rates were also the highest of any sampled aside from dark nitrite oxidation in *D. labyrinthiformis* and dark nitrate reduction in *P. porites* at site 2.

3.2.5. *Acropora palmata*

A. palmata was sampled at site 6 and uniquely did not harbor active DIN cycling across any of the transformations investigated. Before accounting for SW contributions, individual replicates displayed high variability with substantial transformation rates in the surrounding seawater. This resulted in the large margin of error observed for the otherwise negligible ambient light nitrite oxidation rate (0.1 ± 0.1 nmol L^{-1} day^{-1}).

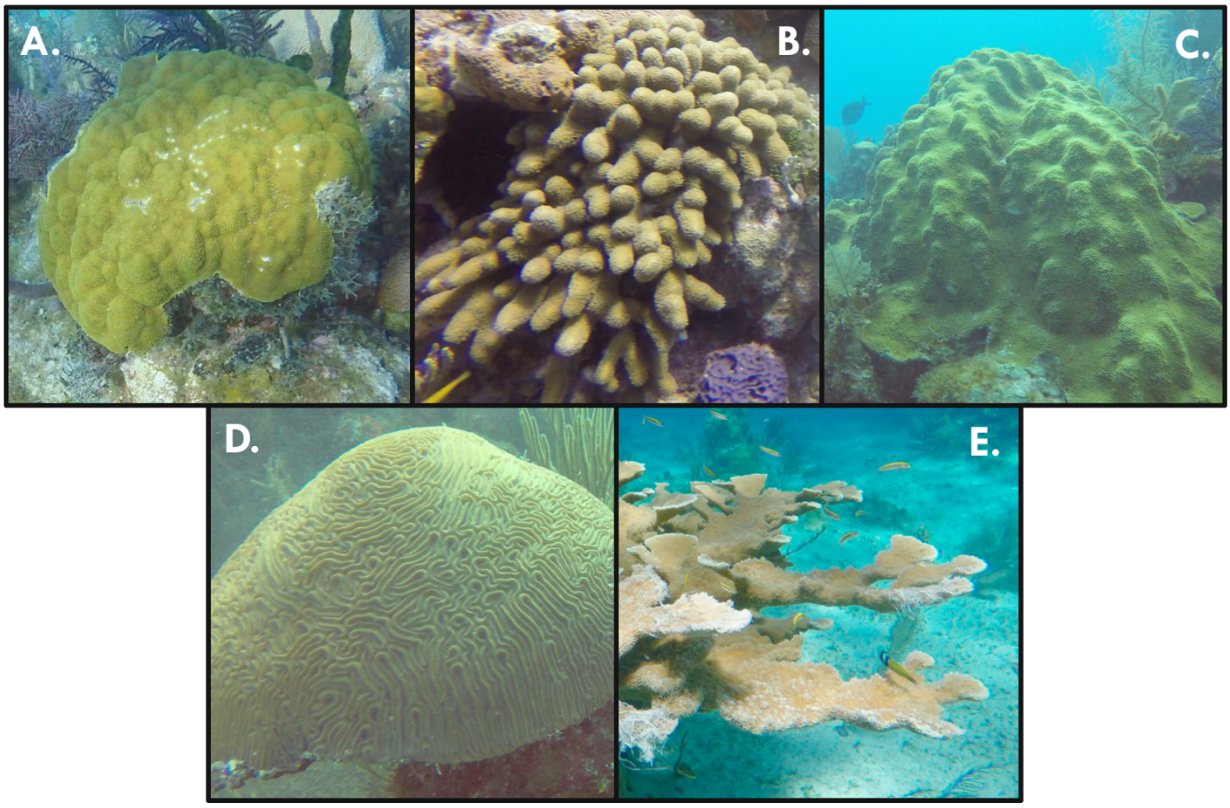

Figure 3.1: Coral species sampled in this study. **A:** The mustard hill coral, *Porites astreoides*. **B:** *Porites porites*, commonly known as finger coral. **C:** *Orbicella faveolata*, formerly classified as *Montastraea faveolata*, or mountainous star coral. **D:** The brain coral, *Diploria labyrinthiformis*. **E:** *Acropora palmata*, or elkhorn coral.

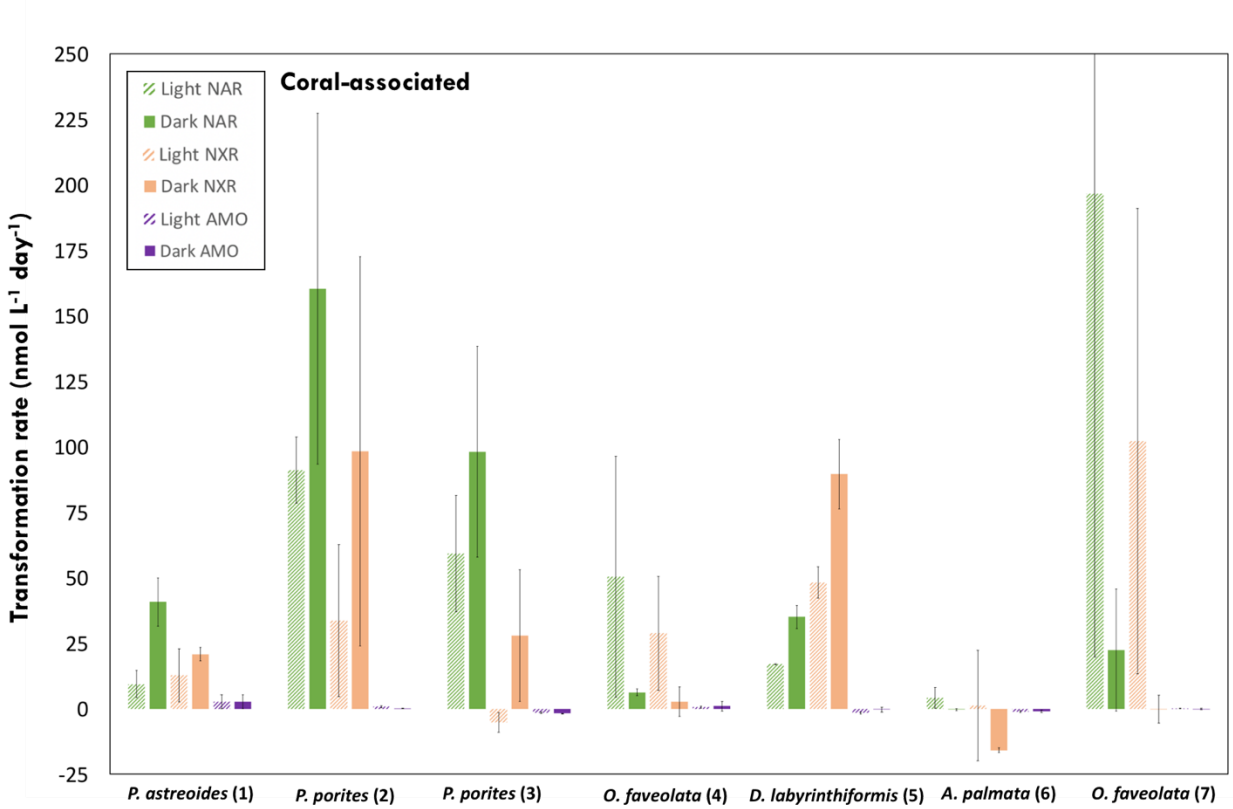

Figure 3.2: Coral-associated nitrate reduction (NAR), nitrite oxidation (NXR), and ammonium oxidation (AMO) rates within dark and light treatments. Rates are reported by site number, in parentheses, and coral species. Error bars show the range of duplicates.

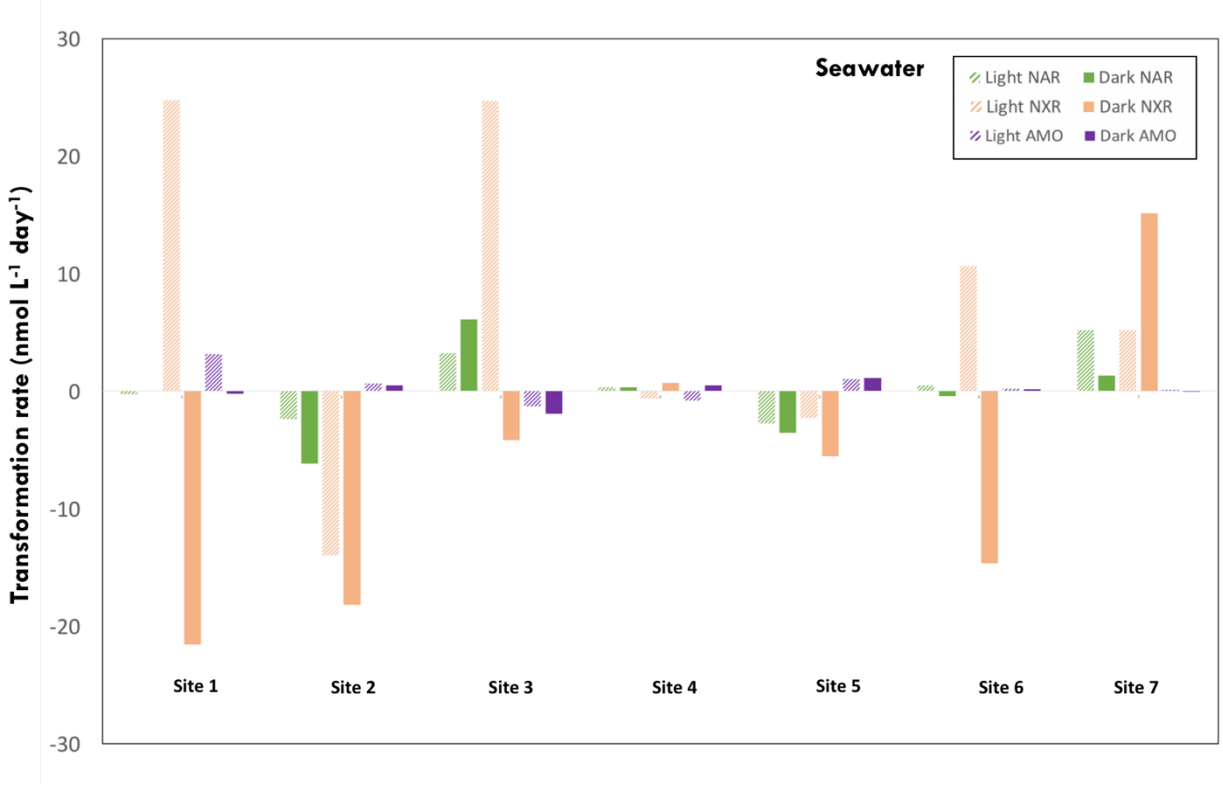

Figure 3.3: Measured nitrate reduction (NAR), nitrite oxidation (NXR), and ammonium oxidation (AMO) rates from seawater only (SW) incubations, reported by site number.

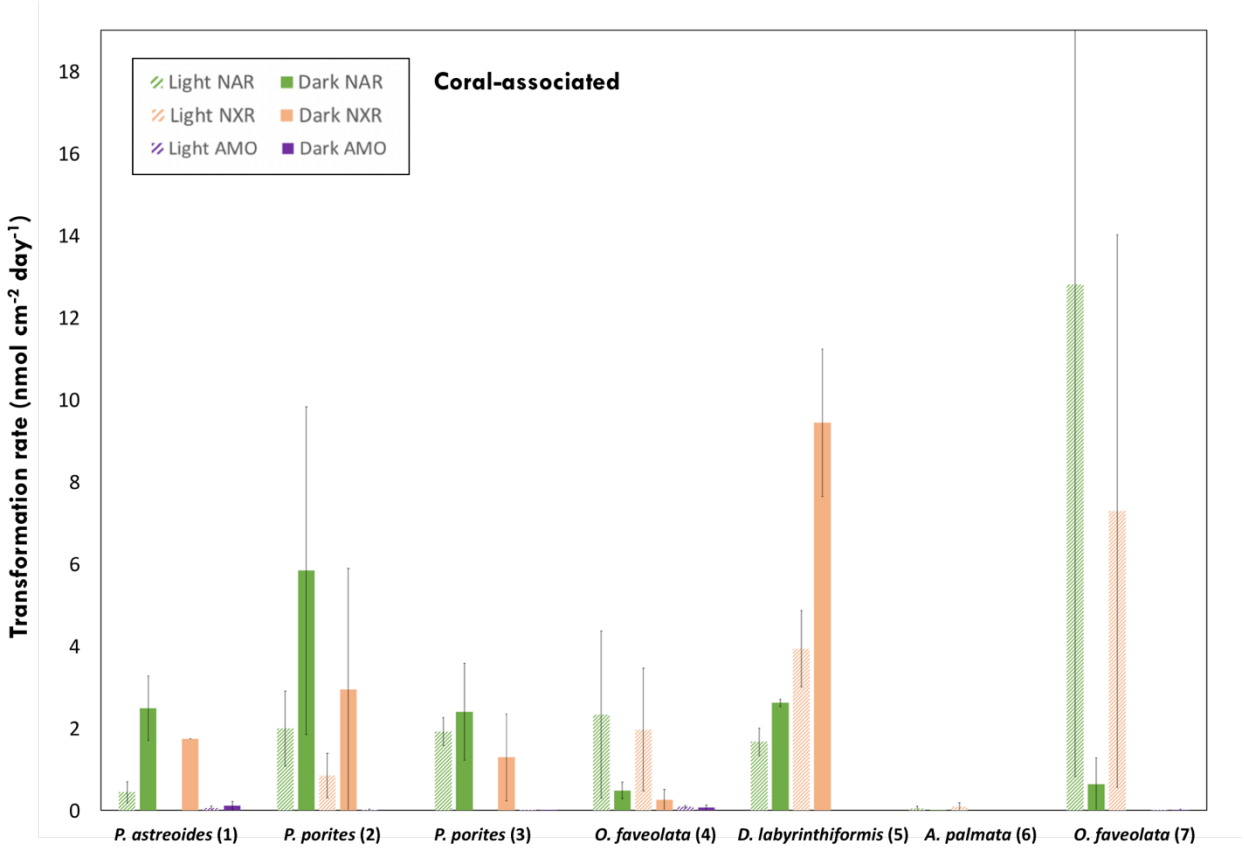

Figure 3.4: Coral-associated nitrate reduction (NAR), nitrite oxidation (NXR), and ammonium oxidation (AMO) rates normalized by the surface area of each coral. Rates are reported by site number, in parentheses, and coral species. These values were calculated according to Equation 2.1. Error bars show the range of duplicates.

3.3. N_2O and N_2 production rates

Production of $^{15}N_2O$ and/or $^{15}N_2$ from our tracer experiments provides a smoking gun of fixed N loss from the system, from canonical denitrification, nitrifier denitrification, and/or anammox. Production of ^{15}N-N_2O was detected from incubations inoculated with $^{15}NO_3^-$ and $^{15}NO_2^-$ (**Figure 3.5**), but not from those inoculated with $^{15}NH_4^+$. N_2 production rates were analyzed, but signals were less than the high detection limit of our methodology. The only conclusion is that, broadly, denitrification and anammox rates were less than 40-200 nmol cm^{-2} day^{-1} based on the range of tissue surface areas sampled (or less than 1 μmol L^{-1} day^{-1} non-normalized). This does not mean that these metabolisms were not present, as the other transformation rates (including N_2O production) were well below this threshold (20-70 nmol L^{-1} day^{-1} for nitrate reduction and 10-80 nmol L^{-1} day^{-1} for N_2O production), but that our experimental design was not sensitive enough for detection of ^{15}N-N_2 production.

Seawater only (SW) rates were again detected for $^{15}N_2O$ production, and in some cases were larger than their corresponding coral incubations (as in the cases of $^{15}NO_2^-$ additions in *P. porites* and in the light $^{15}NO_2^-$ addition for *A. palmata*). This exclusively occurred for $^{15}NO_2^-$ additions and implies the variable ability of the ambient reef water to produce N_2O. Further analysis of the reef microbial community would be necessary to elucidate this trend, but during normalization calculations, any rate less than 0 nmol cm^{-2} day^{-1} was assumed to be zero. Based on the apparent negative rates calculated for the SW incubations, it is evident that the detection limit based on our experimental methodology was 10 nmol L^{-1} day^{-1} of N_2O production for $^{15}NO_2^-$/$^{15}NO_3^-$ additions and 2 nmol L^{-1} day^{-1} for $^{15}NH_4^+$ additions.

3.3.1. *Porites astreoides*

For *P. astreoides*, only minor coral-associated $^{15}N\text{-}N_2O$ production from $^{15}NO_3^-$ and $^{15}NO_2^-$ additions was detected, but was low (<1 nmol cm^{-2} day^{-1}) after accounting for the high SW contribution in the light $^{15}NO_2^-$ addition (~15 nmol L^{-1} day^{-1}).

3.3.2. *Porites porites*

$^{15}N\text{-}N_2O$ production was observed for *P. porites* from both light and dark $^{15}NO_3^-$ treatments at site 2 and only from the dark $^{15}NO_3^-$ treatment at site 3. $^{15}N\text{-}N_2O$ production was detected from the seawater surrounding *P. porites* (10-63 nmol L^{-1} day^{-1}) at both sites, and was higher in the ambient light treatment. After correcting for these SW contributions, only the $^{15}NO_3^-$ treatments produced discernible N_2O (<1 nmol cm^{-2} day^{-1}).

3.3.3. *Diploria labyrinthiformis*

D. labyrinthiformis produced substantial $^{15}N\text{-}N_2O$ from injections of both $^{15}NO_3^-$ and $^{15}NO_2^-$ and hosted the greatest rates of $^{15}N\text{-}N_2O$ production of any species (8.2 ± 2.4 nmol cm^{-2} day^{-1} for the dark $^{15}NO_2^-$ addition). N_2O production rates were in excess of ambient SW N_2O production rates for both sets of ^{15}N tracer inoculations. Dark rates were higher than light rates in both cases, before and after normalization. Normalized N_2O production rates were higher for $^{15}NO_2^-$ additions (2.8 ± 0.1 nmol cm^{-2} day^{-1} in the light and 8.2 ± 2.4 nmol cm^{-2} day^{-1} in the dark) than for $^{15}NO_3^-$ additions (2.1 ± 1.0 nmol cm^{-2} day^{-1} in the light and 3.8 ± 2.7 nmol cm^{-2} day^{-1} in the dark).

Notably, an equivalent number of moles of $^{15}NO_3^-$ and $^{15}NO_2^-$ that was transformed in the nitrate reduction experiments was also converted to $^{15}N\text{-}N_2O$. For $^{15}NO_3^-$, ~2-3 nmol N cm^{-2} day^{-1} was reduced to $^{15}NO_2^-$ (**Figure 3.4**) and ~2–4 nmol N cm^{-2} day^{-1} of N_2O was produced from $^{15}NO_3^-$ injections (**Figure 3.7**). Thus, within the margin of error of our measurements, NO_3^- to NO_2^- to N_2O was quantitatively converted.

3.3.4. *Orbicella faveolata*

^{15}N-N_2O production was detected for light and dark $^{15}NO_3^-$/$^{15}NO_2^-$ additions, and all coral-associated N_2O production rates were roughly statistically equivalent ($\leq 1 \pm 0.5$ nmol cm^{-2} day^{-1}) after normalization. The SW N_2O production rate from the $^{15}NO_2^-$ incubation in ambient light at site 7 was surprisingly high, despite low SW N_2O production in all other *O. faveolata* incubations.

3.3.5. *Acropora palmata*

^{15}N-N_2O production was observed for *A. palmata* in the dark $^{15}NO_2^-$ incubation and both ambient light and dark $^{15}NO_3^-$ additions. However, these rates were only slightly greater than the ambient SW rates and so were low after normalization and correction for SW contributions (<1 nmol cm^{-2} day^{-1}). The rates of transformation for the other cycling pathways were negligible. Surprisingly, $^{15}N_2O$ production in the seawater surrounding *A. palmata* was substantial for both the ambient light and dark $^{15}NO_2^-$ treatments (30 nmol L^{-1} day^{-1} and 40 nmol L^{-1} day^{-1}, respectively). For the ambient light $^{15}NO_2^-$ incubation, this N_2O production by the surrounding seawater occurred even in the absence of any coral-associated $^{15}N_2O$ production.

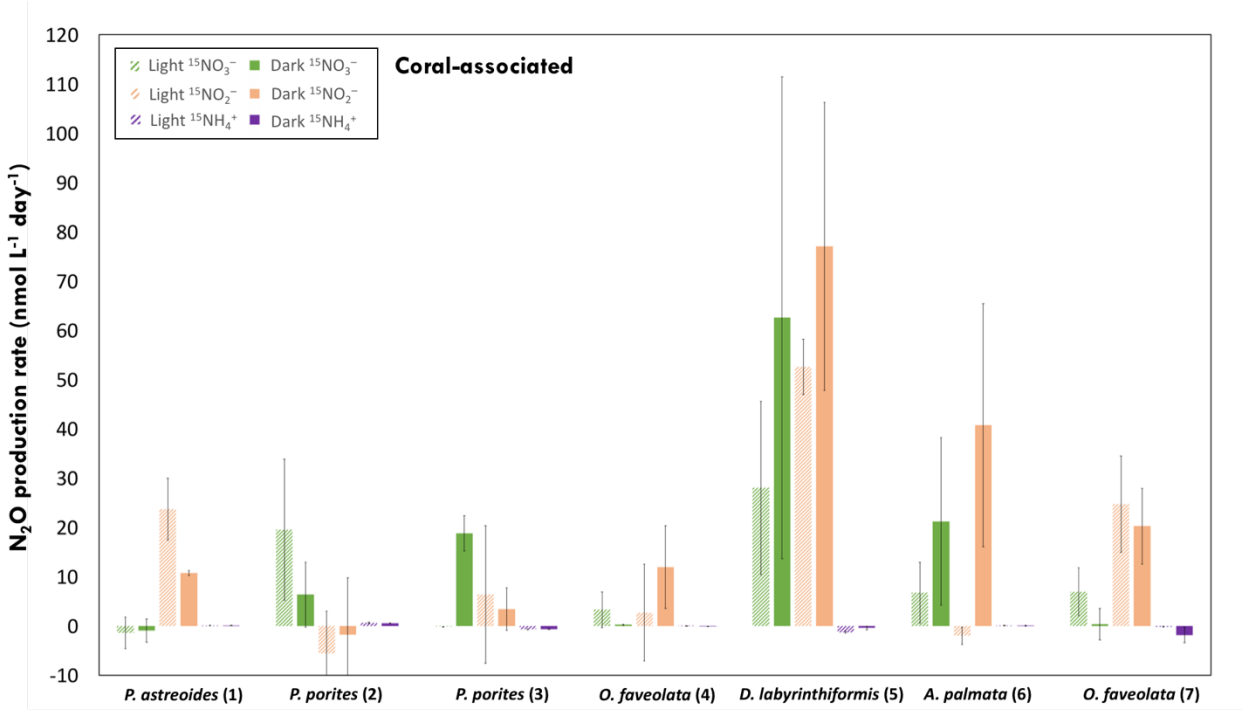

Figure 3.5: Coral-associated N_2O production rates for each ^{15}N tracer inoculation for coral incubations. Rates are reported by site number, in parentheses, and coral species. Error bars show the range of duplicates.

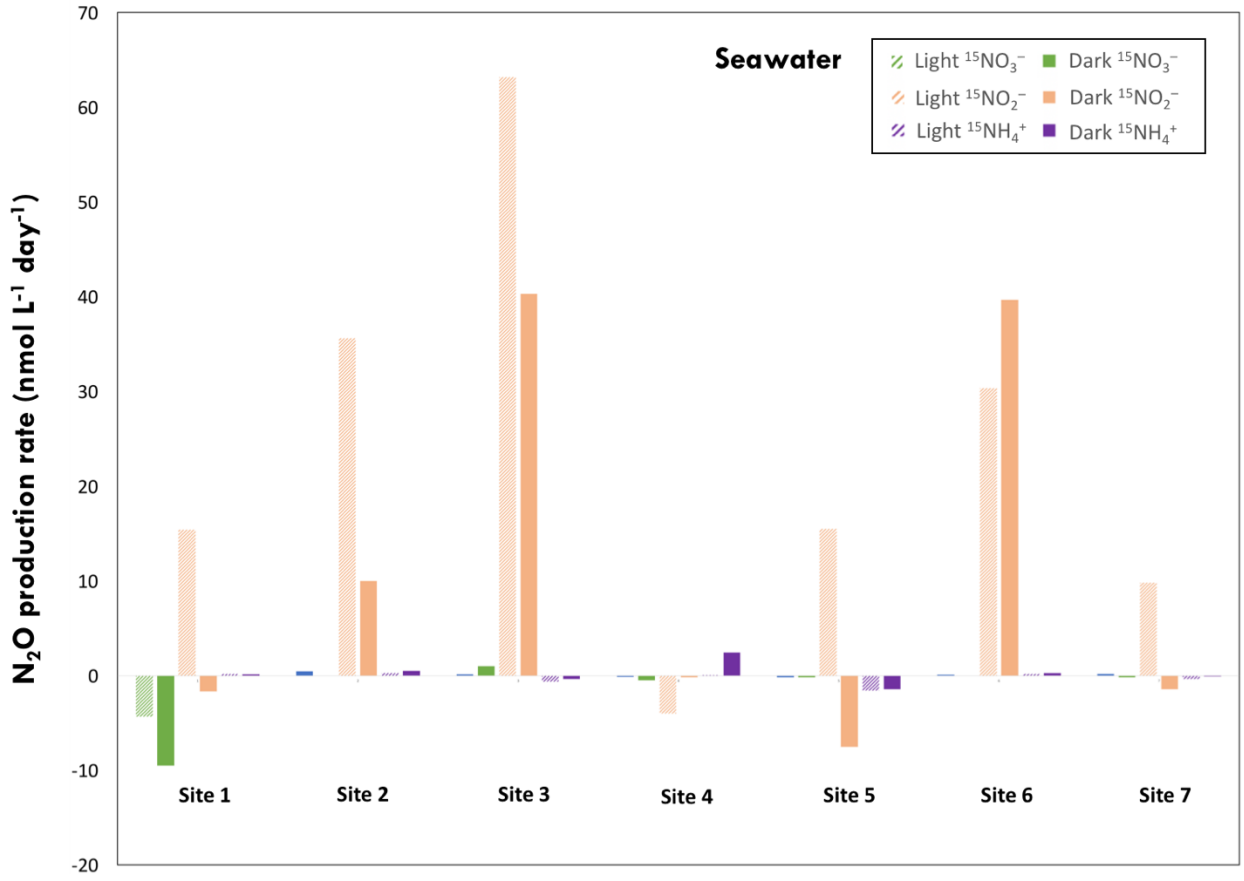

Figure 3.6: Measured N_2O production rates for each ^{15}N tracer inoculation of seawater only (SW) incubations, reported by site number.

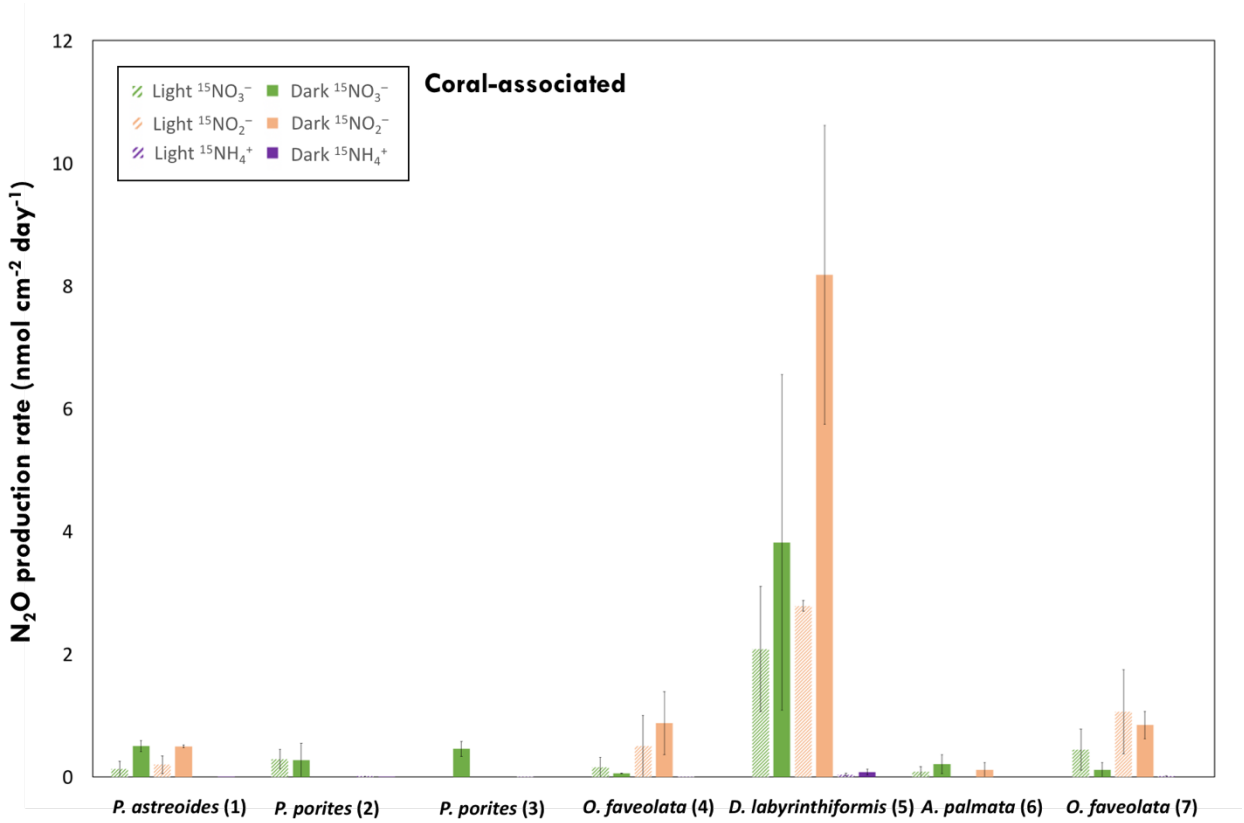

Figure 3.7: Coral-associated N_2O production rates for each ^{15}N tracer inoculation normalized by the surface area of each incubated coral. Rates are reported by site number, in parentheses, and coral species. These values were calculated according to Equation 2.1. Error bars show the range of duplicates.

4. Discussion:

4.1. Nitrate reduction and denitrification

The suite of coral incubations detected nitrate reduction to nitrite, which had previously only been inferred to occur in tropical reef-building corals based on the presence of denitrifying genes in the coral microbiome (Kimes *et al.*, 2010; Yang *et al.*, 2013). In the global oceans, nitrate reduction is an important source of nitrite for nitrogen loss metabolisms via its subsequent reduction. As nitrate reduction is also the first step for the pathways of NO_3^- assimilation and dissimilatory reduction of NO_3^- to ammonia, and because more organisms are capable of nitrate reduction than of complete denitrification (Zumft, 1997), the discovery of nitrate reduction does not on its own imply the presence of a larger suite of nitrogen loss metabolisms. However, the additional detection of $^{15}N\text{-}N_2O$ production from incubations inoculated with $^{15}NO_3^-$ and $^{15}NO_2^-$ confirms at least the further reduction of nitrate and nitrite to N_2O (**Figure 4.1**). This mechanism of nitrogen loss via reduction to nitrous oxide is an important indicator of the potential for canonical denitrification, although our N_2 production measurements were constrained by the high detection limit of our study design (1 μmol L^{-1} day^{-1}) and so could not directly detect a rate for this process. Nevertheless, these nitrate reduction and N_2O production data provide strong evidence that denitrification is active among tropical reef-building corals with nitrate reduction rates up to 13 nmol cm^{-2} day^{-1} and N_2O production rates up to 9 nmol cm^{-2} day^{-1}.

The nitrate reduction rates measured in Gardens of the Queen corals are substantially lower than those measured in coastal marine sediments. In sediments, the absence of O_2 beyond a few millimeters in depth affords the anoxic conditions necessary for anaerobic nitrate reduction and denitrification (Revsbech *et al.*, 1980). In coastal sediments, nitrate reduction of 7.2 μmol cm^{-2} d^{-1} has been measured, which is an order of magnitude larger than the highest nitrate reduction rates measured in these Gardens of the Queen corals (Laverman *et al.*, 2006). The disparity is smaller for

unconsolidated reef sediments, which have displayed nitrate reduction rates of 20 nmol cm^{-2} d^{-1} and denitrification rates of 2.4 nmol cm^{-2} d^{-1} (Capone *et al.*, 1992). Unlike in sediments, on coral reefs anaerobic conditions are likely ephemeral given that the surrounding waters are oxygenated. Thus, coral-associated denitrifiers may exist within localized anoxic environments inside of the coral; microscale measurements of O_2 concurrent with rate measurements are necessary to definitively assess the relationship between O_2 and nitrate/nitrite reduction in corals.

The rates for N_2O production observed in this study agree with previous measurements of N_2O dynamics measured above coral reef ecosystems. Extrapolating from a representative N_2O production rate of 1 nmol cm^{-2} day^{-1} over the globally estimated 250,000 km^2 of reef area (Spalding and Grenfell, 1997), reefs could contribute 4400 metric tons per annum of nitrous oxide to the atmosphere. Previous studies on lagoonal reef ecosystems in Australia have estimated a potential contribution of 6500 metric tons per annum based on N_2O outgassing above coral reef waters using time series air-flux observations via cavity ring down spectroscopy (CRDS) (O'Reilly *et al.*, 2015). That these distinct methods yield such similar estimations of coral reef N_2O outgassing is striking, and lends credibility to our rates being close to those found *in situ* rather than having been affected by sampling artifacts. Reefs, however, are minor contributors to the estimated annual 2-10 Tg of N-N_2O released to the atmosphere by the global oceans (Codispoti, 2010; Ciais *et al.*, 2013). The N_2O contribution from reefs is also likely highly variable and localized because of differences between coral species-associated microbial communities and, thus, their denitrifying potentials.

4.2. Nitrification

Preliminary genetic surveys have suggested that there is potential for oxidation of ammonia to nitrite in tropical corals, as *amoA* sequences have been found in the mucus of *Favia granulosa* corals (Beman *et al.*, 2007; Siboni *et al.*, 2008). Prior to this study, no direct rate measurements had been published on corals, although ammonium oxidation had previously been found to be below detection

in ambient oligotrophic surface waters (Newell *et al.*, 2013) like those in which tropical corals reside. In all species, ammonium oxidation rates were low in comparison to other nitrogen transformations. Dilution of the $^{15}N\text{-}NH_4^+$ pool by introduction of $^{14}N\text{-}NH_4^+$ via nitrogen remineralization (ammonification), which would minimize the apparent ammonium oxidation rate, can be discounted as a source for these low rates because remineralization is too slow (<1 nmol N L^{-1} reef water day^{-1}) to completely obscure ammonium oxidation (McNally *et al.*, 2017).

The $^{15}N\text{-}NH_4^+$ incubations acted as an analog for the introduction of ammonium to coral reef ecosystems by fish and other types of reef life. On reefs, ammonium – which is directly and sporadically excreted by fish (Randall and Wright, 1987) – provides up to 42% of the nitrogen that is eventually assimilated into organic tissue (Grover *et al.*, 2008). This uptake is rapid, with a pulse of 20 μmol L^{-1} ammonium being used by the coral ecosystem in less than an hour (Pernice *et al.*, 2012). The $^{15}N\text{-}NH_4^+$ introduced during these experiments was not observed in the N_2O pool nor as nitrite from ammonium oxidation, which implies the preferential incorporation of the NH_4^+ amendments into coral and symbiont cells. In other words, the NH_4^+ amendments were likely assimilated, leaving little for oxidation to nitrite. This could underscore the importance of ammonium excretion by fish in recycling N in coral reef ecosystems, but measurements of ^{15}N within the coral tissue should be carried out to confirm these ammonium assimilation dynamics in a more targeted way.

Nitrite oxidation to nitrate was detected at rates generally lower than those of nitrate reduction except for incubations of *D. labyrinthiformis*, for which the reverse was observed. Nitrite oxidation rates were also elevated for incubations of *Porites* sp., although these were lower and more variable than for *D. labyrinthiformis* and were roughly equivalent to their corresponding nitrate reduction rates. Nevertheless, we might expect to observe a general trend of higher nitrite oxidation rates in the dark considering the inhibition of this process by light.

Both ammonium oxidation and nitrite oxidation, the specific steps of nitrification, are inhibited by exposure to sunlight. Sunlight intensities as low as 3 μmol of photons m^{-2} s^{-1} have been shown to cause a 50 to 70% inhibition of ammonium oxidation after 2 hours of exposure; nitrite oxidation is similarly hindered, although the extent varies depending on the dose and wavelength of light (Guerrero and Jones, 1996a). In the global oceans, it is posited that these light inhibition dynamics might contribute to producing the ubiquitous primary nitrite maximum (PNM), an accumulation of NO_2^- at the base of the euphotic zone (Ward, 2008). The exact mechanism of this feature's formation remains unresolved, but has been attributed to the differential photoinhibition of ammonium oxidation and nitrite oxidation (Olson, 1981). For nitrification, partial recovery from light-induced inhibition has been observed to begin after 2.5–3 hours in dark conditions (Alleman *et al.*, 1987; Guerrero and Jones, 1996b). Thus, the light conditions maintained during these experiments by using dark mesh to approximate ambient light levels at reef depth (80% attenuation) are unlikely to have caused severe photoinhibition.

4.3. Implications for nitrogen fixation

Based on the rates of nitrate reduction and N_2O production observed in this study, the corals' supply of nitrogen would be depleted after 10 days without the presence of an active source; nitrogen fixation likely balances this outflux and has been observed in reef-building corals (Williams *et al.*, 1987), coral-associated cyanobacteria (Lesser *et al.*, 2007), and coral mucus (Grover *et al.*, 2014). If the estimates of nitrate reduction to nitrite in this study proceeded through the complete denitrification pathway, 3-14 nmol N cm^{-2} d^{-1} would be lost. Nitrogen fixation rates in reef-building corals have been observed in excess of this loss rate (24-72 nmol cm^{-2} d^{-1}) in *Acropora* sp. and *Porites lobata* corals (Shashar *et al.*, 1994), although rates are highly species-specific (Lema *et al.*, 2012). Additional sinks for nitrogen have been measured in corals, such as excretion of organic nitrogen up to 100 nmol cm^{-2} d^{-1} in *Acropora cervicornis* (Szmant *et al.*, 1990), so it is reasonable for nitrogen fixation to be in excess of denitrification.

Further, export of excess nitrogen to sediments may serve to fuel the sediment denitrification rates observed in coral reefs (Capone *et al.*, 1992) and the growth that supports biological N transfer from corals and algae to fish.

4.4. Light versus dark reactions

One of the most critical physical variables impacting the coral environment is light. In the presence of light, microalgal symbionts photosynthesize and produce hyperoxic conditions with a strong gradient of oxygen within tissues, reaching >250% of air saturation after a few minutes (Revsbech *et al.*, 1995). Likewise, at night the respiratory activities of host and microbes cause hypoxia and sometimes anoxia, decreasing to <2% of air saturation again on the order of minutes (Revsbech *et al.*, 1995; Agostini *et al.*, 2012). These internal changes promote multiple functional groups of symbiotic microorganisms with different oxygen requirements, specifically those involved in the transformation of nitrogen compounds, to potentially be both spatially and temporally separated within distinct microenvironments of the coral (Fiore *et al.*, 2010; Wangpraseurt *et al.*, 2012).

Although, in general, observed rates of nitrate reduction and nitrite oxidation were higher for dark treatments, *O. faveolata* hosted higher rates for both processes under ambient light and displayed a greatly decreased nitrogen metabolism in the dark. For this species, nitrate reduction occurring in the light could produce additional nitrite to, in turn, continue to fuel nitrite oxidation. This trend of nitrogen cycling activity only in the light condition may point to different photosynthesis/respiration dynamics in *O. faveolata* relative to the other species sampled, or even suggest nitrogen transformations that are primarily mediated by photosynthetic associates.

In *O. faveolata*, phototrophic bacteria such as those of the order *Rhodobacterales* – which have been found to be abundant in mucus from *O. faveolata* colonies in the Florida Keys (Morrow *et al.*, 2012) – might reduce nitrate to nitrite aerobically. This would then provide sufficient nitrite to support the nitrite oxidation metabolism observed, but would favor sunlit conditions. Although the genetic

potential of *O. faveolata* associates to denitrify has not yet been probed, the conspicuous absence of nitrate reduction in the dark condition, as well as this coral's minimal N_2O production, speaks to a limited potential for denitrification. The capacity to denitrify also appears to be limited for *Porites* sp. corals, which showed similarly minimal N_2O production as well as nitrate reduction rates that were not statistically different between light treatments.

4.5. Other variations among species

The mucus, tissue, and skeleton of the coral animal harbor different assemblages of microbial symbionts and are also distinct from the surrounding water masses (Apprill *et al.*, 2016; Pollock *et al.*, 2018). These associations follow species-specific trends, but similar characteristic microbiota are observed within genera (Rohwer *et al.*, 2001, 2002). With these rate data, this pattern seems to broadly hold, with species' nitrogen cycling characteristics exhibiting the same trend throughout multiple trials (in the cases of *O. faveolata* and *P. porites*) and within genera (*P. porites and P. astreoides* exhibit similar nitrate reduction, nitrite oxidation, ammonia oxidation, and nitrous oxide production rates). While admittedly limited by sample size, the similarity for *O. faveolata* and *P. porites* across sample sites – as well as between *P. porites* and *P. astreoides* – suggests that there might be characteristic microbial nitrogen metabolisms that are associated with specific coral genera.

A. palmata was largely inactive in its observed nitrogen metabolisms as compared with the other species sampled. It is also highly susceptible to white pox disease (Miller *et al.*, 2008), a condition induced by the human pathogen *Serratia marcescens* that is transmitted via sewage outflows (Sutherland *et al.*, 2010, 2011; Joyner *et al.*, 2015). *A. palmata* has experienced precipitous declines (>70% coral cover in the Caribbean) due to this affliction (Patterson *et al.*, 1993) and future studies should continue to probe its nitrogen cycling metabolisms to further elucidate this species' apparent sensitivities.

D. labyrinthiformis displays the greatest potential for denitrification. This species exhibited the highest rates of N_2O production (9 nmol cm^{-2} d^{-1} from $^{15}N\text{-}NO_2^-$ inoculations), which are likely also

indicative of elevated rates of subsequent N_2 production. As additional corroboration, each mole of $^{15}NO_3^-$ and $^{15}NO_2^-$ added was subsequently lost as ^{15}N-N_2O, with rates increasing when subjected to dark, presumed-suboxic treatments. In Caribbean coral reef ecosystems, this species has shown resilience to nutrient influxes: *D. labyrinthiformis* is increasing in abundance on eutrophic reefs on Curaçao (Vermeij *et al.*, 2007) and has shown a fourfold increase in larval recruitment rate under elevated nutrient concentrations (a 9% total nitrogen fertilizer spike) relative to ambient conditions (Chamberland *et al.*, 2017). This success stands in stark contrast to other species, like *P. porites* (Marubini and Davies, 1996). The ability to persist despite eutrophic conditions combined with the increased nitrate/nitrite reduction metabolisms observed here strongly suggests a causal link with a potentially increased abundance of denitrifiers in the *D. labyrinthiformis* holobiont relative to other coral species, and could predict its continued success in environments impacted by local anthropogenic effluents.

Although these data directly confirm the potential of coral associates to conduct denitrifying metabolisms, the generalizability of these results is limited by the size of the dataset. Specifically, time and incubation vessel constraints were such that only 2 coral incubation replicates could be collected for each set of conditions and only one set of incubations performed at each site. This limited our ability to test the statistical significance of the measured rates and to apply other statistical and analytical methods. Future studies should aim to expand this experimental setup to introduce additional replicates and thus enable significance tests of the resultant data. Subsequent experiments should also aim to sample more than one colony at a given site in order to facilitate increasingly robust rate measurements of coral-associated nitrogen cycling.

Ambient nutrient concentrations have been shown to impact the microbial communities of corals, with local variations from 5-11 μmol L^{-1} in total nitrogen explaining up to 40% of the taxonomic dissimilarity among dominant genera in a community of the tropical stony coral *Mussismilia*

braziliensis on a single reef (Silveira *et al.*, 2017). The rate data from the present study do display an apparent correlation with ambient DIN concentrations (**Figure 4.2**), although a larger sample size and analysis of the same species across various sites would contribute to explaining this trend more rigorously. *P. porites* and *O. faveolata* were the only species to be sampled at multiple sites, but the corals sampled from sites with lower ambient DIN concentrations did tend to have higher nitrate reduction rates, although these trends were not statistically significant due to high variability between replicates. Nevertheless, it is suggestive that the site with the absolute lowest nitrate concentrations (site 7; 40 nmol L^{-1}) had the absolute highest measured rate of nitrate reduction (24.8 nmol cm^{-2} day^{-1}). Overall, the patterns from these data suggest that differences between species-associated holobionts are a larger factor in corals' nitrogen-cycling potential than ambient environmental conditions, at least for a relatively pristine ecosystem like the Gardens of the Queen.

Importantly, low ambient nutrient concentrations do not also imply low transformation rates; for example, the flux of nitrite through both reductive and oxidative pathways might be large despite its limited standing concentration in coral reef ecosystems. These "cryptic" linked cycles are important for the biogeochemical cycling of other important nutrients (Canfield *et al.*, 2010; Babbin *et al.*, 2015) and in other environments, including closely defined spaces such as marine particle aggregates (Callbeck *et al.*, 2018). This image, of a tightly coupled cycle comprising the flux of vital nutrients despite low standing concentrations, may be an appropriate portrait of coral nitrogen cycling in otherwise oligotrophic reef waters.

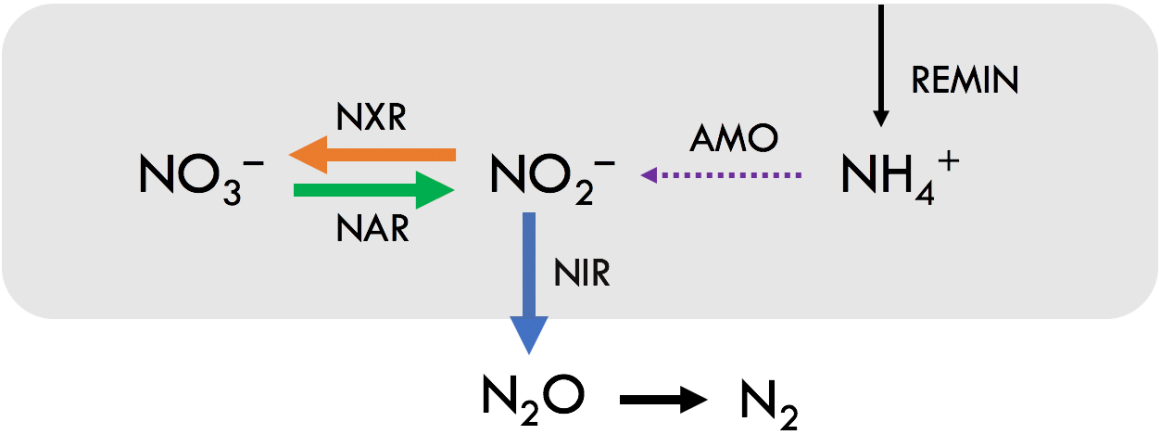

Figure 4.1: System of nitrogen cycling steps examined by this experimental set-up, with cartoon arrows representing their apparent contribution to a baseline tropical coral nitrogen cycle. Nitrate (NO_3^-) reduction to nitrite (NAR), nitrite (NO_2^-) oxidation to nitrate (NXR), and nitrite reduction to N_2O (NIR) displayed detectable rates greater than for ammonium (NH_4^+) oxidation to nitrite (AMO). NIR represents the main sink for nitrogen to leave the system and remineralization represents the primary source.

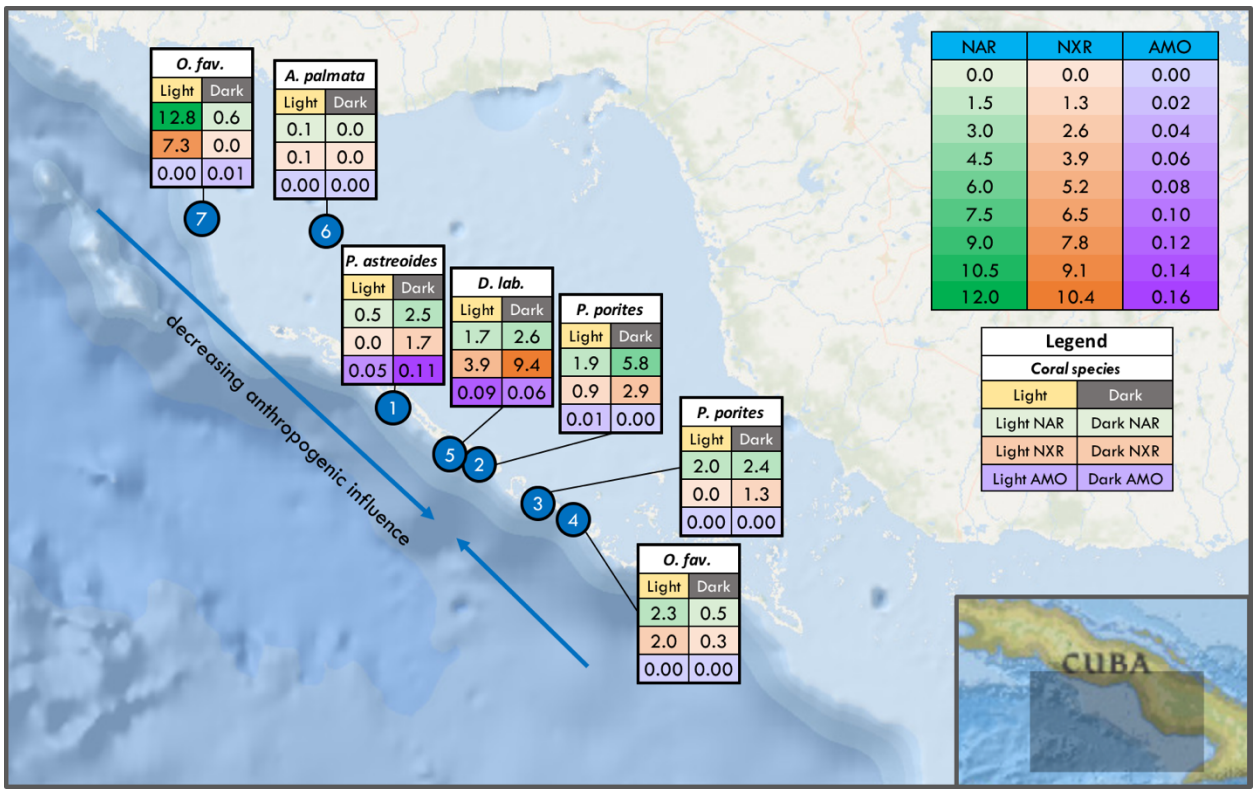

Figure 4.2: Average nitrogen cycling rates (nmol L^{-1} cm^{-2} day^{-1}) for nitrate reduction to nitrite (NAR), nitrite oxidation to nitrate (NXR), and ammonium oxidation to nitrite (AMO) by sample site and light/dark treatments.

5. Conclusions and next steps

This work has contributed the first direct rate measurements of nitrate reduction to nitrite, nitrite oxidation to nitrate, and nitrite reduction to nitrous oxide in five species of Caribbean reef-building corals (**Figure 5.1.**). In summary, *Diploria labyrinthiformis* exhibited the strongest potential for denitrification based on its elevated rates of nitrate reduction and nitrous oxide production, although N_2 production could not be directly detected. *Acropora palmata* displayed little to no active nitrogen metabolism, but both *Porites* corals (*Porites astreoides* and *Porites porites*) showed increased nitrate reduction in the dark conditions with little evidence of further reductive metabolisms. The main nitrogen processing in *Orbicella faveolata* was nitrate reduction to nitrite and nitrite oxidation back to nitrate, which was only detected under light conditions and so was likely linked to phototrophy. Finally, ammonium oxidation rates were scarcely detected, likely due to the efficient assimilation of the added $^{15}NH_4^+$ and light inhibition of nitrification.

Although this study has contributed new rates to the broader picture of nitrogen cycling in corals, these data suggest that it will be worth returning to more precisely measure N_2 production and simultaneous N_2-fixation rates. The N_2O production observed, particularly for *D. labyrinthiformis*, implies significant potential to observe denitrification in reef-building coral species, but a revised experimental scheme is required in order to directly detect these rates of N_2 production. Similarly, future measurements of N_2 production could probe for the presence of anammox in tropical corals with greater resolution. In this study, any anammox production was similarly limited by the N_2 detection limit of our experimental methodology.

In addition, future work should investigate the identities of the microorganisms responsible for the nitrogen metabolisms observed in this study. Although outside of the scope of this thesis, coral tissue pellets were stored for DNA extraction from the Gardens of the Queen corals incubated in these experiments. From these samples, the genetic capacity of the coral-associated microbial

community to carry out nitrogen transformations can be assessed. This information will provide additional evidence for the presence of diverse nitrogen metabolisms in tropical corals, and may suggest additional rate measurements to be made or identify specific coral species with promising nitrogen cycling potential.

The coral-associated nitrogen cycle is complex and dynamic, with chemical and microbial factors that are analogous to features observed in other aquatic systems. The diurnal variations of coral reef oxygen concentrations are perhaps most analogous to those of tidal salt marshes, which also host dramatic daily O_2 variability. This allows for a similar separation of aerobic and anaerobic processes (Smith and Able, 2003), as regular flooding introduces excess organic matter to stimulate respiration and, subsequently, support denitrification rates ranging from 2-12 nmol cm^{-2} day^{-1} (Koch *et al.*, 1992; Bai *et al.*, 2017). Similarly, the co-occurring oxidative and reductive processes within coral microenvironments is akin to that observed in oxygen minimum zones (Babbin *et al.*, 2017) and, based on the evidence presented in this work, seem to harbor a similarly dynamic suite of microbial nitrogen metabolisms. With a more complete image of the baseline coral-associated nitrogen cycle coming into focus, we will be better able to assess its chemical and microbiological similarities to processes in other marine environments and anticipate its response to larger biogeochemical changes.

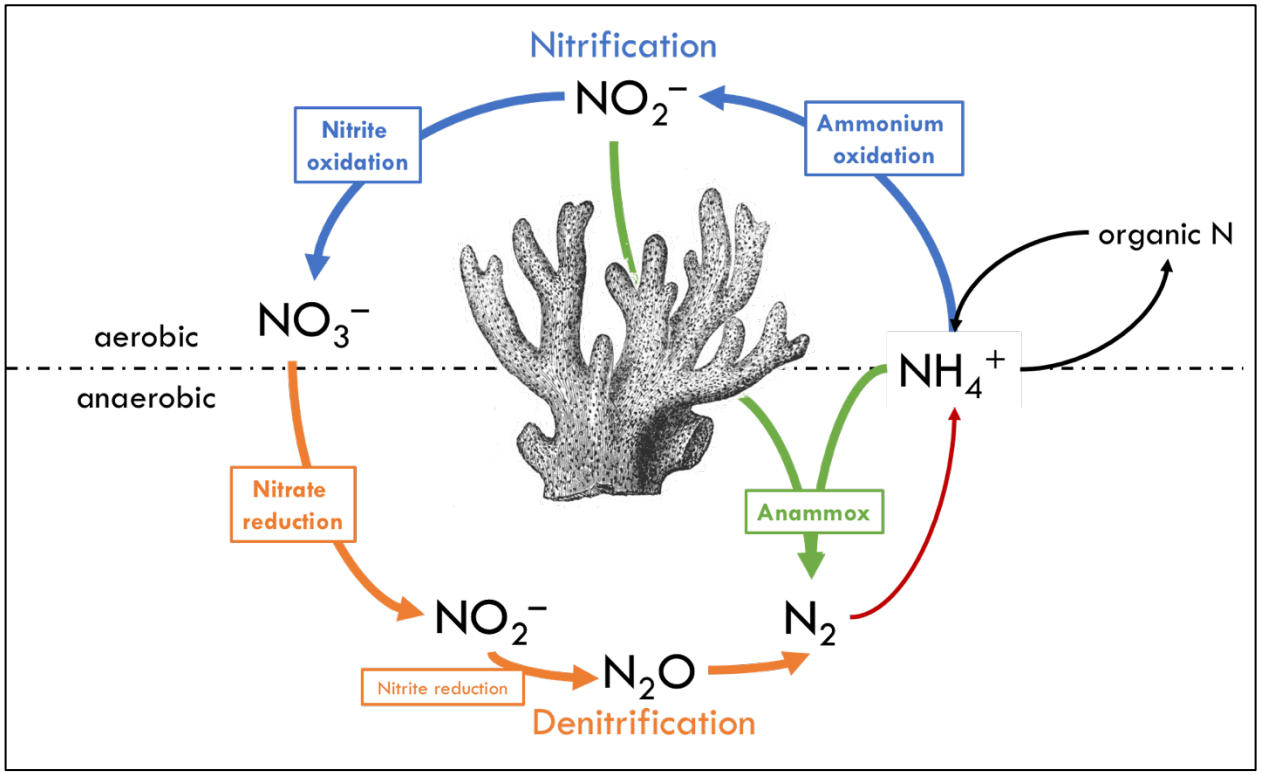

Figure 5.1: Contribution of this thesis towards a more complete nitrogen cycle in tropical reef-building corals (as compared with previous knowledge in **Figure 1.2**). Rates contributed (or, in the cases of denitrification and anammox, capped) by this work are bolded relative to others. Direct rates for nitrate and nitrite reduction, nitrite oxidation to nitrate, and ammonium oxidation were detected and represent novel contributions to the scientific picture of nitrogen cycling in tropical corals. Anammox and denitrification were not directly detected, but the detection limits of our experimental design indicate that they are lower than 1 μmol L^{-1} day^{-1}.

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Appendix

Table A.1: Coral incubation characteristics.

Incubation # (site)	Coral #	^{15}N tracer	Species	Surface Area (cm^2)	Polyp count	Polyp density (polyps/cm^2)	Estimated dry weight (g)	Volume (cm^3)
1 (5B)	1	NO_3^-	*Porites astreoides*	7.1	191	2680	0.19	3.9
	2			8.3	212	2560	0.22	3.4
	3			6.3	182	2870	0.17	2.6
	4			5.2	142	2720	0.13	1.6
	5	NO_2^-		14.3	331	2320	0.22	2.8
	6			11.2	301	2690	0.16	4.1
	7			9.3	247	2660	0.21	2.9
	8			7.8	191	2430	0.20	2.7
	9	NH_4^+		4.5	123	2710	0.11	3.2
	10			6.8	162	2390	0.11	1.1
	11			7.1	187	2620	0.19	1.7
	12			8.8	236	2670	0.19	2.0
2 (6B)	1	NO_3^-	*Porites porites*	25.3	703	2780	0.26	5.9
	2			12.5	351	2810	0.19	2.5
	3			8.1	285	3500	1.35	1.4
	4			18.4	567	3080	0.67	3.0
	5	NO_2^-		21.0	563	2680	0.24	3.6
	6			18.8	524	2790	0.20	4.3
	7			13.9	376	2700	0.22	3.8
	8			8.6	248	2870	0.15	2.1
	9	NH_4^+		7.9	218	2750	0.21	1.5
	10			19.3	589	3050	0.10	4.2
	11			24.2	611	2520	0.21	4.5
	12			21.6	595	2760	0.27	7.8
3 (10B)	1	NO_3^-	*Porites porites*	12.6	362	2870	0.22	2.0
	2			14.6	374	2560	0.35	1.6
	3			11.8	321	2710	0.12	2.1
	4			7.4	211	2870	1.06	1.2
	5	NO_2^-		18.3	533	2920	1.04	3.5
	6			10.4	289	2790	0.21	2.3
	7			6.7	187	2790	0.50	1.1
	8			9.7	264	2720	0.38	1.5
	9	NH_4^+		10.5	312	2970	0.20	1.9
	10			19.1	554	2900	0.10	4.8
	11			15.3	402	2640	0.21	3.8
	12			14.3	397	2780	0.27	4.9
4 (16C)	1	NO_3^-	*Orbicella faveolata*	4.9	22	450	0.20	3.1
	2			7.5	38	510	0.17	4.3
	3			3.6	21	590	0.14	7.2
	4			5.5	31	560	0.18	7.4
	5	NO_2^-		5.6	36	640	0.18	4.7
	6			4.9	23	470	0.47	9.9
	7			5.0	24	480	0.19	4.2
	8			3.5	14	400	0.07	0.9
	9	NH_4^+		5.1	18	360	0.11	7.8
	10			5.3	27	510	0.13	3.9
	11			6.4	38	590	0.15	6.1
	12			5.5	29	530	0.18	5.3

Table A.1, continued: Coral incubation characteristics.

Incubation # (site)	Coral #	Site	Species	Surface Area (cm^2)	Polyp count*	Polyp density* (polyps/cm^2)	Estimated dry weight (g)	Volume (cm^3)
5 (18A)	1	NO_3^-	*Diploria labyrinthiformis*	3.4	nd	nd	0.17	3.2
	2			5.0	nd	nd	0.15	4.9
	3			5.8	nd	nd	0.21	4.7
	4			4.2	nd	nd	0.12	9.8
	5	NO_2^-		5.1	nd	nd	0.19	2.3
	6			4.0	nd	nd	0.04	3.1
	7			3.3	nd	nd	0.14	5.5
	8			3.6	nd	nd	0.06	3.8
	9	NH_4^+		2.9	nd	nd	0.05	1.6
	10			2.2	nd	nd	0.07	1.9
	11			8.0	nd	nd	0.22	10.1
	12			3.9	nd	nd	0.17	2.5
6 (15)	1	NO_3^-	*Acropora palmata*	33.4	287	860	0.25	7.2
	2			26.3	217	830	0.15	5.9
	3			35.8	317	890	0.21	8.3
	4			28.2	231	820	0.12	4.3
	5	NO_2^-		21.9	212	970	0.11	3.9
	6			34.3	298	870	0.20	6.2
	7			37.0	306	830	0.15	5.1
	8			23.0	224	970	0.12	4.0
	9	NH_4^+		22.9	226	990	0.09	3.6
	10			12.2	102	840	0.05	1.1
	11			17.4	147	840	0.10	5.7
	12			17.4	142	820	0.08	2.1
7 (2B)	1	NO_3^-	*Orbicella faveolata*	5.1	33	650	0.12	1.5
	2			6.0	40	660	0.19	2.0
	3			11.7	81	690	0.22	5.7
	4			5.5	36	660	0.12	2.9
	5	NO_2^-		4.8	33	690	0.06	2.2
	6			4.5	31	680	0.06	1.7
	7			9.3	58	620	0.07	5.9
	8			7.7	47	610	0.13	1.8
	9	NH_4^+		6.4	41	650	1.26	1.2
	10			5.2	38	720	0.06	2.9
	11			2.9	21	730	0.03	1.4
	12			5.2	36	690	0.06	0.9

*nd = no data